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REPRODUCTIVE CYCLE, SEX CHANGE, AND MORTALITY DURING BROODING OF A VIVIPAROUS SEA CUCUMBER LEPTOSYNAPTA CLARKI.

BY

MARY ANNE SEWELL

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

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Date: 16/8/93

This thesis is dedicated to my grandmother,

Clara Phyllis Wilson
(1903-1992)

who taught us to dance with our dreams.

ABSTRACT

Reproduction in the viviparous apodid sea cucumber Leptosynapta clarki was studied on the west coast of Vancouver Island, Canada. The reproductive cycle of Leptosynapta clarki is annual with gonad growth during the summer months. Male sea cucumbers spawn during November and December and internally fertilize the oocytes within the ovary. Embryonic development is rapid so that within two weeks fertilized eggs reach the pentactula stage. Pentactulae have an open mouth and anus and actively move in the ovarian tubules feeding on droplets and fluid derived from the resorption of unfertilized eggs. Changes in the ovarian wall during the reproductive cycle were examined using histology and TEM.

Mortality of pentactulae during brooding was measured by counting calcareous rings of dead pentactulae. Pentactulae mortality in Leptosynapta clarki can be extremely high (up to 100%), and questions the assumption that the lowest mortality will be found in viviparous species.

Juvenile Leptosynapta clarki are released from the ovary at a length of 1-2 mm in the early spring (April-May). Laboratory growth experiments and regular field sampling show growth to a mean length of 21.1 mm by the reproductive season in the following November. These Year 1 individuals are all reproductively active as males. In their second year, some individuals continue to reproduce as males, while others undergo protandric sex change to reproduce as females. Analysis of the relationship between size and sex revealed a "critical" size for sex change at a body weight of 200-400 mg with a 1:1 sex ratio above 500 mg total weight. Transitional gonads were observed with previtellogenic oocytes and mature spermatozoa suggesting that the "decision" to change sex is made before reproducing in the current reproductive season.

Leptosynapta clarki shows the three commonly associated traits of small adult size, brooding and hermaphroditism. A test of the allometric hypothesis on the association of small size and brooding found no evidence for scaling constraints on brood size. Sequential hermaphroditism in L. clarki may be a method to reduce inbreeding in a low mobility species.

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This research could not have been conducted without the space and facilities of Bamfield Marine Station. I thank the Director, Dr. J. McInerney, and staff of the Marine Station for assistance during all phases of this research. I am also grateful for financial assistance from Bamfield Marine Station in the form of a Graduate Student Award, and a Sigurt Tveit Memorial Scholarship.

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Finally, thanks must go to Winnie-the-Pooh whose words of wisdom have now helped me through two theses. With apologies to A.A. Milne, I misquote from "The House at Pooh Corner" : "It just shows what can be done by taking a little trouble," said Eeyore. "Do you see, Pooh? Do you see, Piglet? Brains first and then Hard Work. Look at it ! *That's* the way to build a (thesis)," said Eeyore proudly.

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CHAPTER 1

General Introduction.

A. Brooding in marine invertebrates.

Reproductive patterns in benthic marine invertebrates are generally of three main types: 1) planktotrophic larvae which feed during their planktonic period, 2) lecithotrophic larvae which derive their energy from yolk reserves and are independent of larval food, and 3) embryos which are retained by the parent or in benthic egg cases and hatch as benthic juveniles (Thorson 1950). In the latter category, retention of the embryos by the parent is usually known as brooding.

Thorson (1950) in reviewing the literature to that time offered a number of generalizations about reproductive patterns that provide the basis for much of our theory in marine invertebrate larval ecology (Roughgarden 1989, Young 1990). Species with planktotrophic larvae produce many small yolk-poor eggs and have highly variable recruitment patterns. In contrast, embryos with non-pelagic development originate in most cases from large yolky eggs and these species have rather constant populations and regular recruitment (Thorson 1950). Additionally, patterns of reproduction with latitude led Thorson (1950) to propose what has become known as "Thorson's Rule" (Mileikovsky 1971) that polar marine invertebrates primarily have benthic embryonic development without any pelagic larval stages. After extensive research in the Antarctic, Pearse et al. (1991) concluded that pelagic development is as prevalent in the Antarctic as in temperate locations such as southern California. Non-pelagic development or brooding, although common in some groups such as cidaroid and spatangoid echinoids, bivalves and gastropods, may not be as prevalent among shallow-water Antarctic marine invertebrates as previously believed (Pearse et al. 1991).

Brooding as a reproductive pattern is proposed to have numerous consequences for the life history of an organism. Brooding is usually associated with reduced dispersal and gene flow and specifically with greater genetic differences between local populations, greater genetic similarity within local populations, reduced geographic range, and perhaps higher speciation and extinction rates (Strathmann 1990 and references therein). In addition, two characters of the adult have been associated with brooding in marine invertebrates: small size and the tendency towards hermaphroditism. Of these the most striking pattern is the association of small adult size and brooding

which occurs in many, but not all, taxa (Strathmann 1985, 1990). Small adult size is usually associated with lower fecundity, earlier sexual maturation, lower adult longevity, and shorter generation time (Strathmann 1985). The correlation between small size and brooding provides one of the best examples of a link between early life history traits and adult life history traits in marine invertebrates (Strathmann 1985). Both these correlations between brooding and adult characters are discussed in detail in Chapter 4.

B. Brooding in Echinoderms.

In the majority of echinoderms reproduction involves the free-spawning of gametes into sea water, external fertilization, and a planktonic phase of development (Hyman 1955). Brooding, where the embryos or juveniles are associated with the adult during development, while not common is found in representatives of all extant echinoderm classes (Lawrence 1987). Embryos are held externally under or on top of the parent (asteroids, Chia and Walker 1991; crinoids, Holland 1991; echinoids, Pearse and Cameron 1991; holothuroids, Smiley et al. 1991; ophiuroids, Hendler 1991); internally in pouches, chambers or bursae (asteroids, Chia and Walker 1991; crinoids, Holland 1991; holothuroids, Smiley et al. 1991; ophiuroids, Hendler 1991); in the coelom (holothuroids, Smiley et al. 1991) or in the ovary (asteroids, Chia and Walker 1991; concentricycloids, Rowe et al. 1991; crinoids, Holland 1991; holothuroids, Smiley et al. 1991; ophiuroids, Hendler 1991).

However, in contrast to the information available on reproduction in species with pelagic development, little is known about the details of brooding in echinoderms. Only the gross morphological modifications associated with brooding have been systematically studied in ophiuroids (Hendler 1991). No information is available on embryonic development for brooding crinoids or concentricycloids (Holland 1991, Rowe et al. 1991) and there are few detailed reproductive studies of any brooding echinoderm (but see Chia 1966, 1969, McClary and Mladenov 1988, 1989, 1990). This is especially true for viviparous forms.

There has been some confusion in the literature in applying the term viviparity to brooding in echinoderms. The term viviparous has been used to describe intrabursal, intracoelomic or intraovarian brood care in the literal sense of "live" birth and without the connotation of nutrient transfer (Turner and Dearborn 1979). Other scientists have restricted the term viviparous to intraovarian or intracoelomic development (Lawrence 1987) or to intraovarian brooding only (Komatsu et al. 1990). In this research I follow Komatsu et al. (1990) in defining viviparity as where "embryos develop within the gonad

or genital tract" (p. 254). Restricting the definition of viviparity to brooding within the gonad or genital tract reduces confusion by using the definition as applied in vertebrates (Wourms and Callard 1992).

Using this definition there are 13 known viviparous species within the Phylum Echinodermata (Table 1-1). Representatives are found in all classes except the Echinoidea (Table 1-1). Six other ophiuroid species have been characterised as intraovarian brooders but none of these species has been critically examined and viviparity confirmed (Hendler 1991). In the majority of viviparous echinoderms information is restricted to observations of embryos in the ovary or basic descriptions of the embryology of development. There is only one study (Messing 1984) that considers viviparity in relation to other aspects of the reproductive biology of the species.

The present study considers viviparity in the apodid sea cucumber Leptosynapta clarki Heding 1928. This species has been chosen for two reasons. Firstly, in contrast to other viviparous holothurians which live subtidally (Leptosynapta transgressor, Brooks 1973) or in the deep sea (Oneirophanta mutabilis affinis, Hansen 1968, 1975; Taeniogyrus contortus, Ludwig 1898), Leptosynapta clarki can be collected in the intertidal. Local intertidal populations in Grappler Inlet, Bamfield, are large (mean density 169 per m^2, Chapter 6) and readily accessible from Bamfield Marine Station allowing regular sampling. Secondly, some aspects of the reproductive biology are known (see below), so this research could focus on questions relating to the influence of viviparity on the life history and reproductive biology of this species.

C. Reproduction in Leptosynapta clarki.

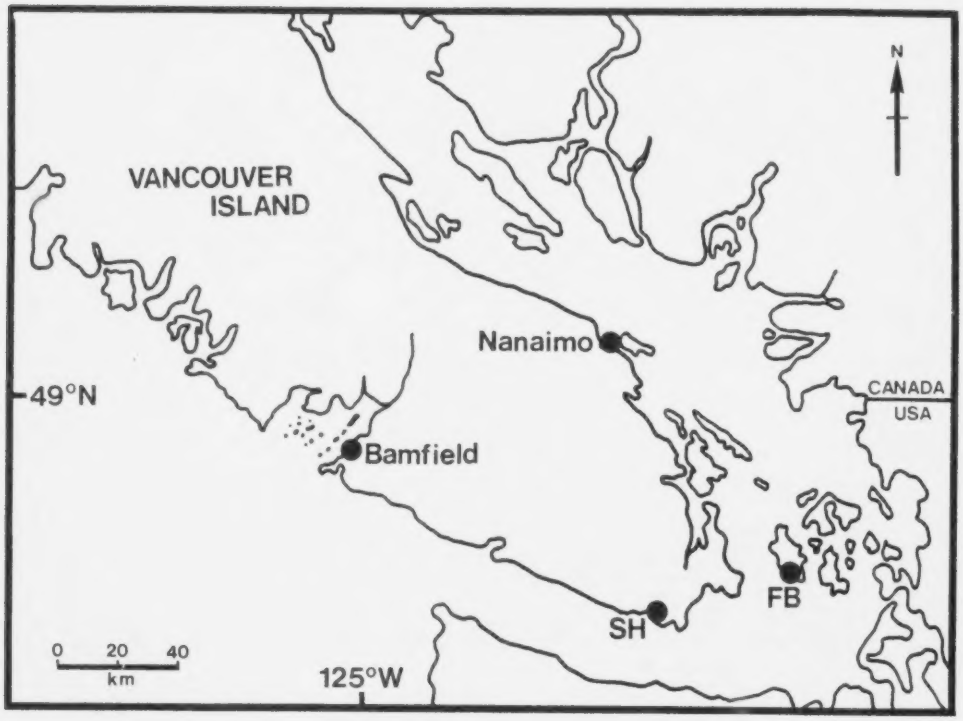
Leptosynapta clarki is found in shallow muddy bays from the Queen Charlotte Islands to central California in the intertidal to depths of 73 m (McEuen 1986, 1987). This species was found to be viviparous and the embryology described by Everingham (1961) at False Bay, San Juan Island, Washington (Fig. 1-1). Some aspects of reproduction in Leptosynapta clarki were also examined by Brooks (1973) at Sooke Harbour, Vancouver Island (Fig. 1-1). Even though these studies were less than 50 kilometres apart, Brooks (1973) described the reproductive period as being four months later than Everingham (1961) and had contradictory results with respect to other aspects of reproduction such as egg size.

Although Everingham provided descriptions of the embryological stages in Leptosynapta clarki many details of reproduction in L. clarki are unknown (Hess et al. 1988). Everingham (1961) himself noted two areas of reproduction that needed to

Table 1-1. Reports of viviparity in the Phylum Echinodermata.

Class	Species	Reference(s)
Asteroidea	Patiriella parvivipara	Keough and Dartnall 1978
	Patiriella vivipara	Dartnall 1969, Chia 1976, Byrne 1991, O'Loughlin 1991
	Asterina pseudoexigua pacifica	Komatsu et al. 1990
Concentricycloidea	Xyloplax medusiformis	Rowe et al. 1988
Crinoidea	Comatilia iridometriformis	Messing 1984
Holothuroidea	Leptosynapta clarki	Everingham 1961
	Leptosynapta transgressor	Brooks 1973
	Oneirophanta mutabilis affinis	Hansen 1968, 1975
	Taeniogyrus contortus	Ludwig 1898
Ophiuroidea	Amphiura microplax	Mortensen 1936
	Amphiura monorima	Mortensen 1936
	Astrochlamys sol	Madsen 1967
	Ophionotus hexactis	Mortensen 1921, Morison 1979

Figure 1-1. Location of collection sites for Leptosynapta clarki on Vancouver Island. Original species description from Nanaimo (Heding 1928); other descriptions or studies from False Bay (FB, Everingham 1961), Sooke Harbour (SH, Brooks 1973) and Bamfield (present study).


N
VANCOUVER
ISLAND
Nanaimo
49°N
CANADA
USA
Bamfield
FB
SH
0 20 40
km
125°W

be resolved. Firstly, the finding of abundant sperm in the ovaries of Leptosynapta clarki sampled in March or April (Everingham 1961, Eckelbarger and Young 1992) led Everingham to suggest that this species might be a protandric hermaphrodite. While protandry might occur in this species, the complete sequence of sexual changes in the adults is unknown (Everingham 1961). Secondly, details of fertilization and the mechanism of sperm transfer are unknown (Everingham 1961, McEuen 1987, Hess et al. 1988). Everingham (1961) suggested that Leptosynapta clarki might be self-fertile, but electrophoretic studies suggest that cross-fertilization is the normal pattern of fertilization (Hess et al. 1988). In addition, there is no information on events occurring within the gonads prior to fertilization and the fate of the juveniles once they leave the mother.

D. Species definition.

Leptosynapta clarki was originally described by Heding (1928) from specimens taken in Roxton Passage, Nanaimo, British Columbia (Fig. 1-1) as being bright yellow in colour and up to 25 cm in length. During the course of my research significant differences in the colour, spicule size, and number of digits on the tentacles were found between specimens collected at Bamfield and False Bay (Fig. 1-1), and the original species description (Heding 1928). Differences were also found between the original species description and specimens of Leptosynapta clarki from Sooke Harbour, Victoria (Fig. 1-1, Brooks 1973), and False Bay (Everingham 1961).

In collaboration with Dr. Ahmed Thandar, University of Durban-Westville, South Africa, specimens of Leptosynapta clarki from Bamfield have been examined in further detail. A number of anomalies have been found between these specimens and the original species description (Heding 1928), leaving some doubt about the species definition of the apodid sea cucumbers found at Bamfield. Differences between Leptosynapta clarki from different geographic locations may simply be attributable to local variation between distantly separated populations of the same species. To determine if there are large differences in morphology between locations, the type specimens for Leptosynapta clarki have been requested from the Copenhagen Museum and when available will be compared to specimens from sites at Bamfield and southern Vancouver Island.

For the purposes of this thesis the apodid sea cucumber found on the intertidal mudflats of Grappler Inlet, Bamfield, is designated as Leptosynapta clarki. Further collaboration with Dr. Thandar upon completion of the thesis will determine if the

Bamfield specimens are Leptosynapta clarki or a previously undescribed species. Changes to the species name will then be made prior to publication of this research.

E. Objectives of the Research.

The objective of this research was to expand on the description of embryology (Everingham 1961) in Leptosynapta clarki by considering events within the gonads prior to fertilization and to give a detailed description of the reproductive cycle of an intertidal population of Leptosynapta clarki (Fig. 1-2) at Bamfield. Specifically the questions addressed were: 1) during what period of the year are the gonads growing and pentactulae brooded in Bamfield? 2) how are the eggs fertilized? 3) is there a change in gonad structure during the transition from oocyte production to brooding of embryos? 4) how is nutrition provided to the pentactulae after the yolk reserves are exhausted? 5) is there any mortality of pentactulae during the brooding period? 6) how are pentactulae released from the mother and do they disperse after release? 7) how fast do the juveniles grow and are they protandric?

While the answers to these questions are important to understanding the reproductive biology of Leptosynapta clarki, they are also of considerable significance to our understanding of viviparity in echinoderms and marine invertebrates in general. Long-held assumptions about the evolutionary significance of viviparity as a reproductive pattern are currently untested because of the limited information we have about reproduction in viviparous species. For example, Thorson (1950) believed that embryonic mortality in viviparous species was extremely low to counteract the low number of yolk-rich eggs produced. As it is generally believed that factors affecting larval mortality are important in the evolution of the general pattern of reproduction (Vance 1973), the finding that mortality may be extremely high in brooded Leptosynapta clarki pentactulae (Chapter 5) is of great significance to current thinking about viviparity as a reproductive strategy.

F. General Organization.

This thesis has been written in the paper-format and consists of five chapters (Chapters 2-6) that have been written as manuscripts intended for submission to refereed journals, a general introduction (Chapter 1), and a general discussion (Chapter 7). As each chapter has been written as a separate manuscript, there is by necessity some duplication in the methods and references in each chapter.

Figure 1-2. Juvenile Leptosynapta clarki collected from intertidal mudflat in Grappler Inlet, Bamfield, British Columbia. Length of individual ca. 4.5 mm.

Throughout the thesis consistent terminology is used to describe the life history stages in Leptosynapta clarki. Hatched embryos within the ovary are referred to as pentactulae. Once these pentactulae are released from the female into the adult habitat they are known as juveniles. Because the juvenile phase in Leptosynapta clarki is completed in 6 months (Chapter 6), to differentiate between these reproductively active post-juveniles and previously reproductive adults, juveniles of the year are referred to as Year 1 males.

Chapter 2 provides the basic descriptive information on the reproductive cycle essential for understanding the complex pattern of reproduction in Leptosynapta clarki. Using histology and observations from fresh material I describe gametogenesis, embryonic development, and the changes in gonad structure during protandric sex change. In this chapter I also describe how the ovarian wall of Leptosynapta clarki undergoes complex changes in the period prior to fertilization and during the early part of the brooding period. These changes to the ovarian wall are examined using transmission electron microscopy in Chapter 3.

In Chapter 4 I examine the association of small size, brooding and hermaphroditism in Leptosynapta clarki. The effects of protandric sex change at the population level are described by extensive sampling of sea cucumbers of a large size range. I also test the allometric hypothesis linking small size and brooding in marine invertebrates (Strathmann and Strathmann 1982). Chapter 5 extends the theoretical context of Chapter 4 by examining the observed mortality in brooded pentactulae of Leptosynapta clarki in terms of our long-held assumption that embryonic mortality is low in viviparous species. Chapter 6 uses laboratory growth rates and field sampling to describe birth, recruitment and growth once released from the mother. In the general discussion (Chapter 7) I summarize the conclusions reached in Chapters 2 to 6 and suggest areas for further research.

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CHAPTER 2

Reproduction of the intraovarian brooding apodid Leptosynapta clarki in British Columbia.

A. Introduction.

Apodid holothurians comprise approximately 18% of the 1250 species of sea cucumbers and have representatives in inshore (Pawson 1982) and deep-sea faunas (Billett 1991). Reproductively the apodids show a diversity of patterns that is unequalled in the other five orders of holothurians (McEuen 1986). Development may occur pelagically or by internal brooding in the coelom or ovary (McEuen 1986). Detailed studies on gametogenesis are limited to a hermaphroditic species Leptosynapta tenuis (Green 1978) and the dioecious species Rhabdomolgus ruber (Menker 1970). Brief details on reproduction are provided for the non-brooding Chiridota laevis (Murdoch 1984) and Opheodesoma spectabilis (Berrill 1966), several deep-sea apodids (Gage et al. 1985), and the brooding Leptosynapta clarki (Everingham 1961, Brooks 1973), Leptosynapta transgressor (Brooks 1973) and Synaptula hydriformis (Estabrooks 1984). However, at this time there is no detailed reproductive study on a brooding apodid that considers both gametogenesis and embryonic development in the reproductive cycle.

Leptosynapta clarki is an intraovarian brooding apodid sea cucumber found on the intertidal mudflats of Grappler Inlet, Bamfield, British Columbia, Canada. This sea cucumber is found at high density on both the intertidal (mean 169 per m^2, Chapter 6) and subtidally (max. 63.2 per m^2, Rumrill 1987). Preliminary details of gametogenesis are provided in an embryological study of Leptosynapta clarki at False Bay, San Juan Island, Washington (Everingham 1961) and in a taxonomic study at Sooke Harbour, Vancouver Island (Brooks 1973). However, many details of reproduction in Leptosynapta clarki remain unknown (Hess et al. 1988). The present study expands on Everingham's description of embryology in Leptosynapta clarki by considering events within the gonads prior to fertilization and gives a detailed description of the reproductive cycle of an intertidal population of Leptosynapta clarki. Gonad indices and histology are used to describe reproduction and the results compared to reproduction in non-brooding holothurians.

B. Materials and Methods.

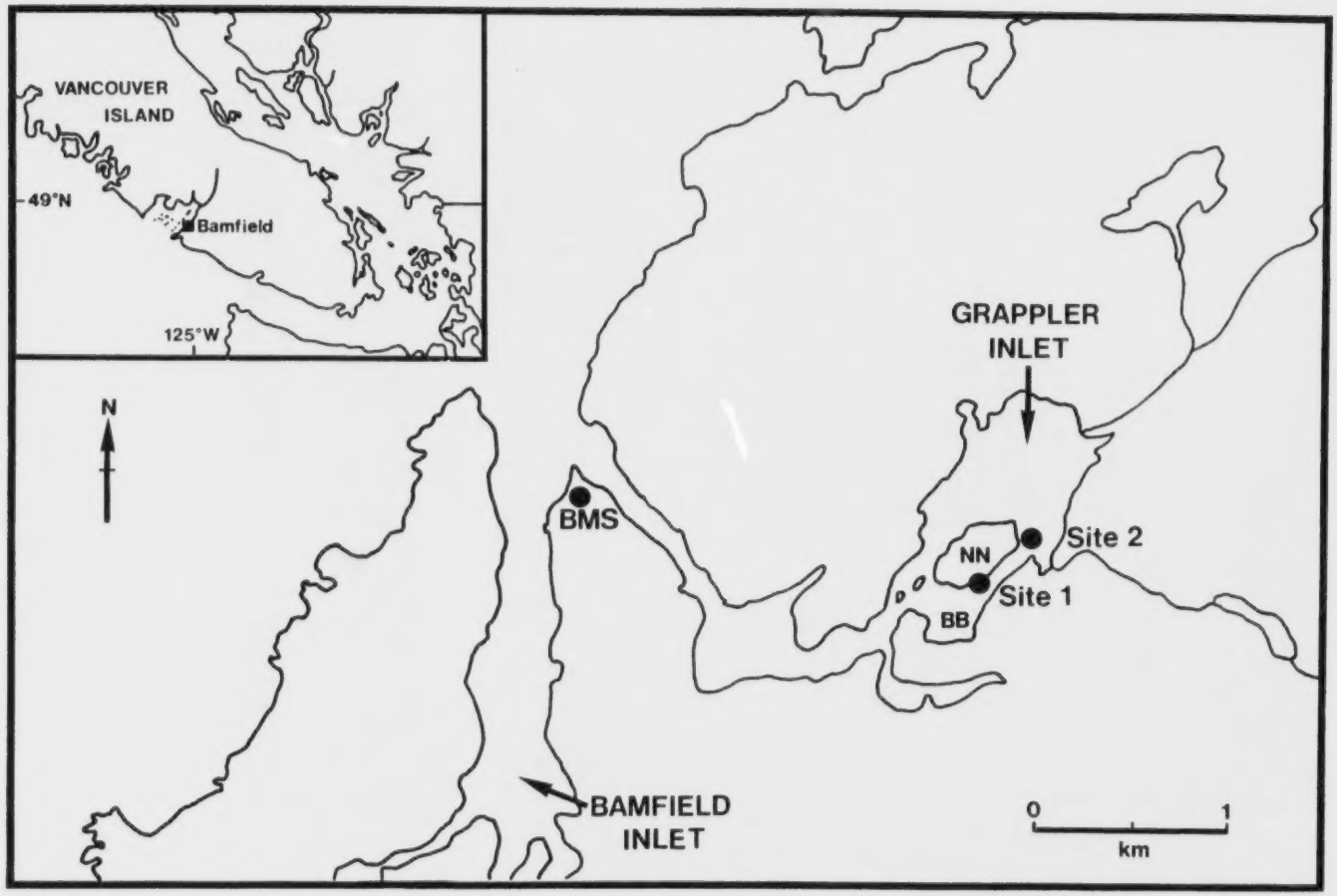
Reproduction of Leptosynapta clarki was studied at two sites on the mudflats of Grappler Inlet, Bamfield, British Columbia (Site 1: 48°49'57" N, 125°06'45" W; Site 2: 48°50'N, 125°06'36" W, Fig. 2-1). Samples were taken from Site 1 on the northern side of Barge Bay (Fig. 2-1) from July 1989 to July 1991. This site has a short intertidal area (ca. 20 m) and a high proportion of gravel in the substrate. From August 1989 reproductive samples were also collected at a second site at the opposite end of the channel separating "No-name" Island from Vancouver Island proper (Site 2, Fig. 2-1). Site 2 is a gentler sloping mudflat (intertidal area ca. 60 m), has a lower proportion of gravel in the substrate and contains Leptosynapta clarki of a larger size than at Site 1 (mean total weight ± SD for all gonad index samples: Site 1: 307.8 ±153.99 mg, N=720; Site 2: 490.4 ± 268.80 mg, N=660). Both sites have extensive Zostera sp. beds in the lower intertidal that extend into the shallow subtidal.

Leptosynapta clarki were collected in the mid-intertidal throughout each site at 4 to 6 week intervals. Animals were collected by sweeping away the upper surface of the mud which exposed the sea cucumbers in their burrows. The sea cucumbers were collected with the surrounding mud and transported to the laboratory.

Leptosynapta clarki were removed from the sediment using a 0.85 mm sieve and placed in a bowl of sea water. Thirty sea cucumbers that were complete and had not autotomised their posterior ends were chosen at random from the bowl and relaxed in 2.5% $MgCl_2$ in sea water (w/v) for approx. 10 minutes. After relaxation each sea cucumber was placed on a tissue to remove external water, and weighed on a fine-scale balance (= total weight in mg). A longitudinal incision was then made from the base of the calcareous ring to the posterior, the coelomic water was drained from the body cavity and the sea cucumber was reweighed (= drained weight).

Sea cucumbers were dissected in sea water under a binocular microscope. The two gonad tubules were removed and weighed for calculation of the gonad index (GI: as a percentage of drained weight). Mean gonad indices were calculated separately for male and female sea cucumbers. As the sex ratio in Leptosynapta clarki is skewed towards males (ca. 3:1, Chapter 4), variable numbers of male and female sea cucumbers were present in each sample. The number of males used in gonad index calculations ranged from 13-28 (mean= 20) at Site 1 and 7-25 (mean=17) at Site 2. The number of females used in calculation of sample means were lower than in males and ranged from 2-17 (mean= 10) at Site 1 and 5-23 (mean=13) at Site 2.

Figure 2-1. Location of sampling sites (Sites 1 and 2) for Leptosynapta clarki in Grappler Inlet, Bamfield, British Columbia. Inset shows location of Bamfield on Vancouver Island. BB= Barge Bay, BMS= Bamfield Marine Station, NN= "No-name" Island.


VANCOUVER
ISLAND
49°N
Bamfield
125°W
N
GRAPPLER
INLET
BMS
Site 2
NN
Site 1
BB
BAMFIELD
INLET
0
1
km

Several modifications in the general use of the gonad index were required for this study: 1) The mean gonad index for females in the brooding period includes all females irrespective of whether they were brooding pentactulae; 2) In the period of sex change, when new eggs began to appear in the gonad tubules, an arbitrary definition of sex was made. Gonads with only a few eggs are referred to as transitional gonads and defined as male. Gonad tubules packed with eggs are included in the female mean; 3) After spawning some resorbed gonads were too small to dissect and weigh. In these sea cucumbers the sex was assumed to be male and an arbitrary value for the GI of 0.01% was assigned.

After weighing, the gonad tubules were placed on a glass slide (38 x 75 mm) with a small amount of sea water and temporarily mounted with a coverslip (35 x 50 x 0.02 mm). Under the microscope the weight of the coverslip revealed details of gonad development, such as the presence and stage of embryos or the initial stages of egg development, without damaging the tubule. The gonad was then preserved in Bouin's fixative for 2 weeks and transferred to 70% ethanol for storage.

Histological sections were prepared for 5 males and 5 females from each site in samples between July 1989 and August 1990. The entire gonad was dehydrated in an ethanol series, embedded in Paraplast, and sectioned at 7µm. Sections were routinely stained in Harris's haematoxylin and eosin. To determine the beginning of vitellogenesis, 40 additional female sections were stained with haematoxylin/periodic acid Schiff (PAS). Sections were examined under a light microscope and each sea cucumber assigned a "stage of maturity" from I to V based on the method of Tanaka (1958). A maturity index was calculated for each sex using the formula of Yoshida (1952) based on the number of individuals in each stage present in each sample:

$$MI = \frac{1(SI) + 2(SII) + \ldots n(Sn)}{N}$$

Where: SI = number of individuals in Stage I.
SII = number of individuals in Stage II.
Sn = number of individuals in Stage n (i.e. Stages III - V).
N = total number of individuals staged in that sample.

In calculation of the maturity index SI includes sea cucumbers in both the post-spawned (i) and post-spawned (ii) stages.

In February 1990 ovaries containing pentactulae and previtellogenic oocytes were preserved in osmium tetroxide for transmission electron microscopy (TEM). Details of preservation are provided in Chapter 3. The ovaries were embedded in Epon resin

(Polysciences), stained for 10 minutes in alcoholic uranyl acetate, followed by 10 minutes in lead citrate and examined on a Phillips E.M. 201 electron microscope.

A brooding index (percent of females brooding pentactulae) was calculated based on the number of females dissected for gonad indices as described above (Sites 1 and 2 combined) and from dissections to examine embryonic development (Chapter 5). The brooding index (%) at each time is therefore based on a variable number of females (range 2-59, mean= 24).

C. Results.

1. General observations.

The ovary or testis of Leptosynapta clarki is located in the anterior coelom and consists of two gonad tubules, one to each side of the dorsal mesentery as in other synaptids (Hyman 1955). Each tubule is attached at the basal end to a small swelling, the gonad basis, that connects the two tubules through the mesentery to the gonoduct. The gonoduct leaves the gonad basis at the anterior end and runs within the mesentery to a single gonopore. Short lobes branch off the main tubule only along the side furthest from the dorsal mesentery; i.e. towards the body wall. Mature ovaries are up to 1 mm in width and a yellow or pink colour. Testes were usually longer and thinner than ovaries and cream to white in colour.

The reproductive cycle in this species is centred around the attainment of ovaries packed with mature oocytes (Fig. 2-2). This state can be achieved by two means: the regrowth of ovaries after functioning as a female (Fig. 2-2C, 2-2D), or protandrous sex change from a male (Fig. 2-2B).

Juvenile sea cucumbers are released from the ovary into the adult habitat in the spring (April-May) at 5-6 months of age, and mature as males in the November following their birth (Fig. 2-2A, Chapter 6). After spawning the testis becomes thin and yellow in colour. The tubules shrink in size towards the gonad basis, and in some specimens the gonad tubule is only present as an extremely small red-pigmented bud on the gonad basis (Fig. 2-2A). These sea cucumbers are presumed to remain as males for the next reproductive season (Fig. 2-2A). Alternatively, after some shrinkage small eggs appear at the end of the tubule adjacent to the gonad basis. These tubules then grow in length because of the continued growth of female tissue at the gonad basis (Fig. 2-2B). Thus some of the male population become females 10-12 months after birth.

Female sea cucumbers undergo two routes to the mature ovary. In this case, however, the pattern depends on whether pentactulae were brooded during the autumn-

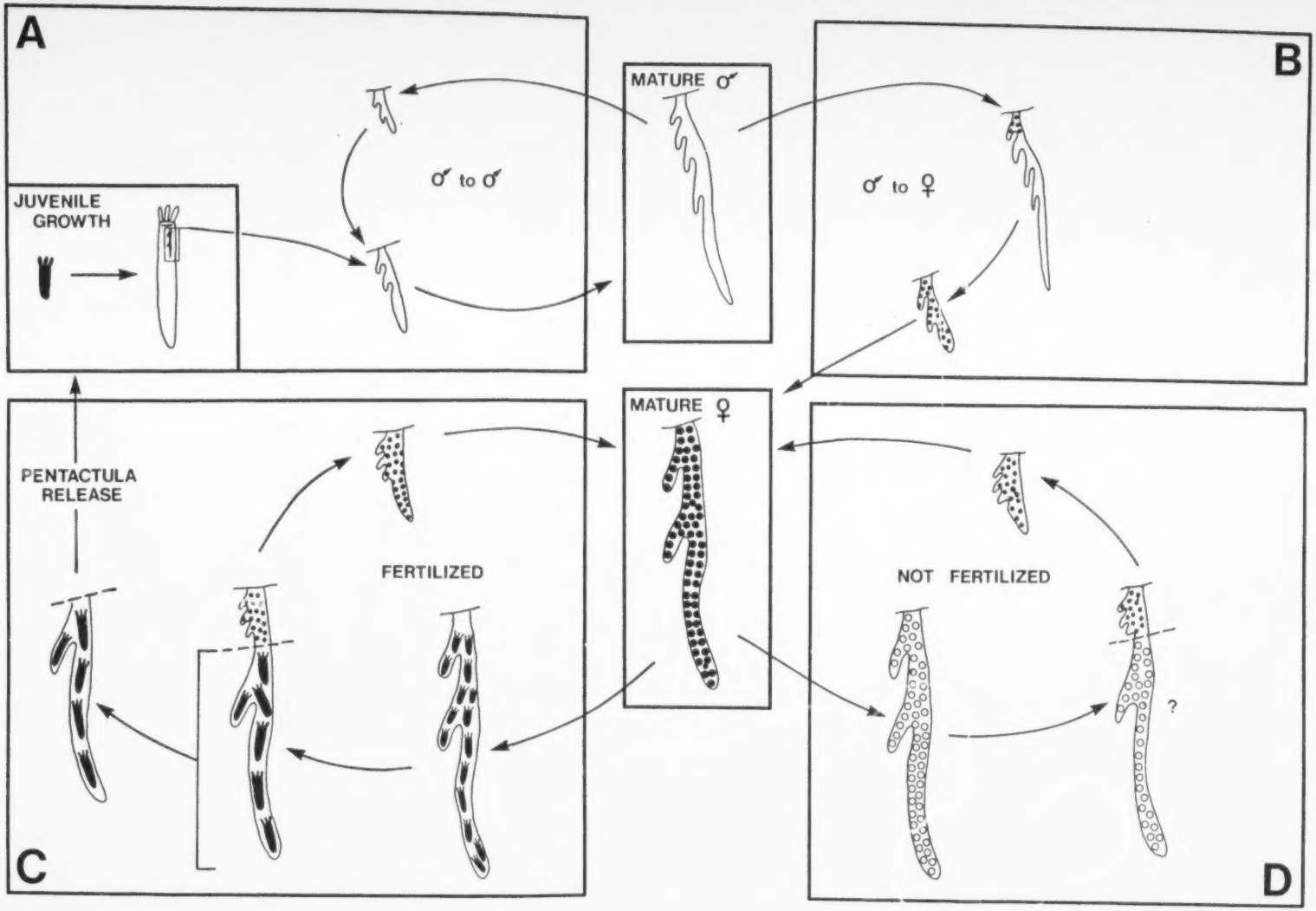
Figure 2-2. Schematic diagram to show patterns of reproduction in Leptosynapta clarki. Information compiled from dissections and histology. Only the left tubule, without the gonad basis, is shown in each case. Details are provided in text.

A. Male to male.

B. Male to female = protandry.

C. Fertilized female to female.

D. Unfertilized female to female.


A
JUVENILE GROWTH
♂ to ♂
MATURE ♂
B
♂ to ♀
MATURE ♀
PENTACTULA RELEASE
FERTILIZED
C
NOT FERTILIZED
?
D

winter period (Fig. 2-2C, 2-2D). Ovaries that were not fertilized are resorbed, and new eggs grow at the basal end as in the male sex-changing gonad (Fig. 2-2D). The ovaries that were fertilized incubate their embryos in the distal end of the gonad, and grow new eggs at the basal end (Fig. 2-2C). Prior to release of the pentactulae from the coelom a break point occurs in the area between the brooding portion of the ovary and the basal region of renewed growth (Fig. 2-2C). The posterior distal part becomes loose in the coelom and pentactulae are released from the body cavity by autotomy or rupture of the body wall (Chapter 6). The basal portion of the ovary with new eggs grows in length to eventually form the mature ovary.

The sex ratio in Leptosynapta clarki is skewed towards males, with the breeding sex ratio ranging from 71-89 % male (Chapter 4). The sex ratio does not reach 1:1 until total weight of the individual is in excess of 500 mg (Chapter 4). Three simultaneous hermaphrodites were found during dissections. In two specimens (31 Aug. 1989) a knot occurred midway along the length of the gonad tubule. Distal to the knot the tubules contained developing oocytes, while the basal end contained male tissue. In the remaining specimen (30 Nov. 1990) there was no knot in the tubule, but the male tissue was again at the basal end. These observations suggest that sex change is reversible as the most recent growth was male, while the older distal growth was female.

2. Gametogenesis.

Gametogenesis was described in Leptosynapta clarki using histological sections. The descriptions provided here are based on those sea cucumbers whose gonads were large enough for dissection and histological processing. Observations on fresh material have also been included in the following descriptions. Ultrastructural details of spermatogenesis in Leptosynapta clarki are described in Atwood (1974a).

a. Male.

Recovery (II).

Recovery gonads were very small (1-2 mg, Table 2-1) and often had a thick eosin-staining tubule wall with haematoxylin-staining nuclei (Fig. 2-3A). A single layer of spermatogonia, with perhaps some spermatocytes, lined the empty tubule lumen (Fig. 2-3A).

Growth (III).

In the growth stage the tubule wall became thinner with reduced eosin-staining (Fig. 2-3B) and the testis weight doubled from the recovery stage (Table 2-1).

Table 2-1. Mean gonad weights (mg) for male and female Leptosynapta clarki in each maturity stage at Sites 1 and 2, Grappler Inlet. Values for post-spawning males include protandric gonads until May 1990; hereafter these were considered as female recovery gonads. Means for Post-spawning females include fertilized and unfertilized ovaries.

	SITE 1			SITE 2		
MATURITY STAGE	MEAN	SE	N	MEAN	SE	N
Male						
Recovery (II)	1.52	0.21	19	2.15	0.46	11
Growth (III)	4.44	1.19	7	4.64	1.19	7
Mature (IV)	8.01	1.20	19	28.59	5.20	11
Shedding (V)	12.60	2.56	9	32.4	9.35	7
Post-spawning (I)	2.50	0.33	18	7.72	2.19	27
Female						
Recovery (II)	2.02	0.48	10	5.3	4.5	2
Growth (III)	9.57	1.06	18	17.62	3.24	16
Mature (IV)	17.53	2.31	8	76.98	14.96	6
Prefertilization (V)	24.56	6.24	8	57.02	9.28	9
Post-spawning (I)	95.39	20.83	20	86.10	12.31	24

Figure 2-3. Histological sections of male Leptosynapta clarki in all maturity stages. All sections except H stained with haematoxylin/eosin.

A. Recovery stage testis with spermatogonia in a single layer. Note thickened eosin-staining tubule wall and haematoxylin-staining nuclei (arrow). Bar= 25 µm.

B. Growth stage testis with convolutions developing in tubule wall. Spermatocyte development primarily occurs at the base of the convolutions. Bar= 25 µm.

C. Mature stage testis with extensive spermatocyte layer (sc). A few spermatids are visible in testis lumen. Bar= 25 µm.

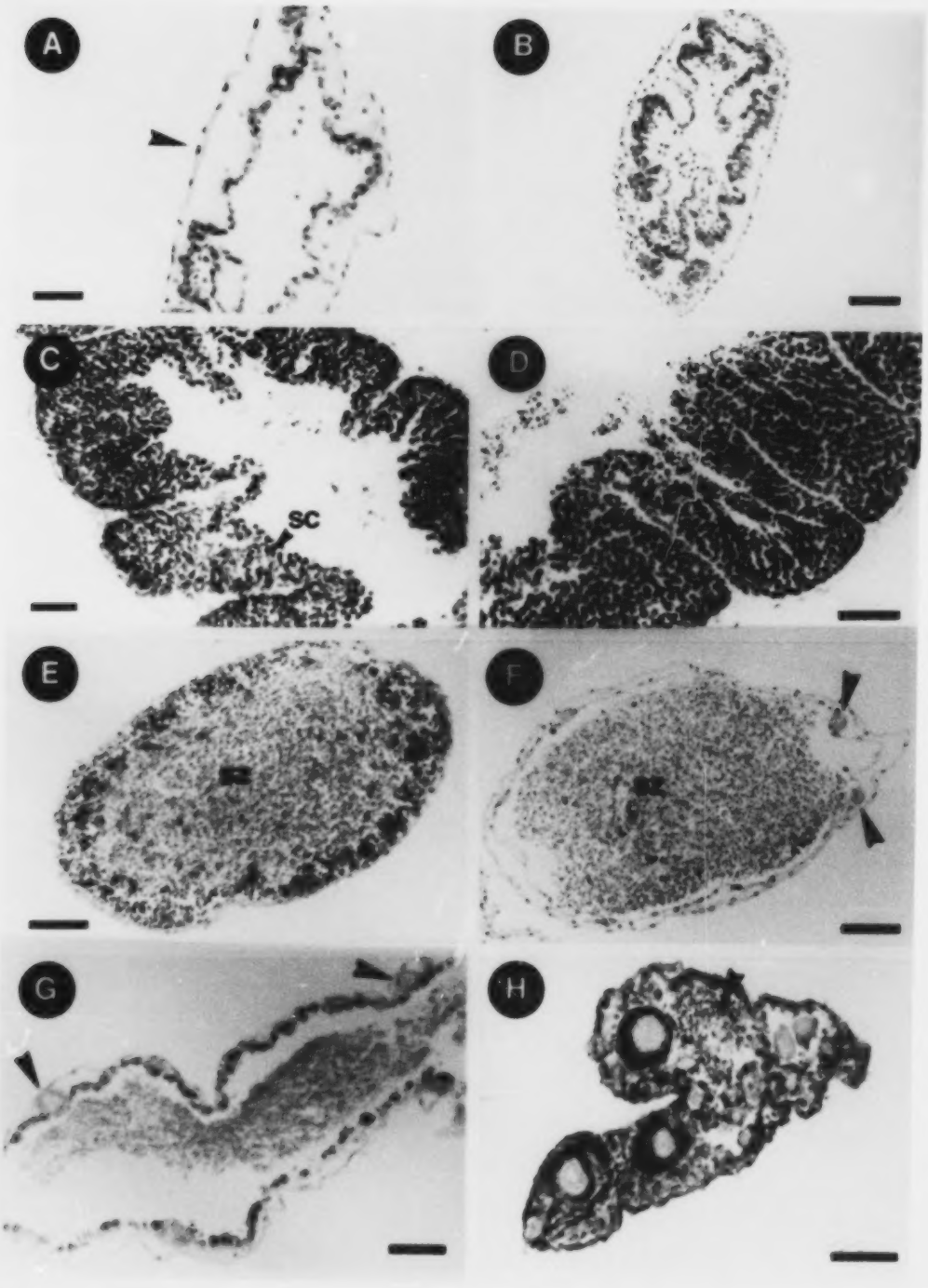
D. Mature stage testis with active development of spermatids in converging columns. Bar = 25 µm.

E. Shedding stage testis. Some spermatocytes remain along the tubule wall. Lumen filled with spermatozoa (sz). Bar= 50 µm.

F. Shedding stage testis with spermatozoa (sz) in the lumen and early oocytes (arrow) forming in germinal epithelium of the tubule wall. Bar= 50 µm.

G. Post-spawned testis in transitional stage. Remnant spermatozoa are present in the lumen and larger oocytes (arrows) are found along the tubule wall. Bar = 50 µm.

H. Post-spawned testis in transitional stage. A few remnant spermatozoa (arrow) are seen in the lumen. Larger oocytes beginning vitellogenesis are found in the tubule. Section stained with haematoxylin/PAS. Bar = 50 µm.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Convolutions began to appear in the germinal epithelium (Fig. 2-3B). There was active spermatocyte development along the tubule wall and an empty tubule lumen (Fig. 2-3B).

Mature (IV).

Mature gonads had a thick layer of spermatocytes extending from the germinal epithelium with a reduced lumen (Fig. 2-3C). Remnants of the convolutions of the growth stage are seen as infoldings of the tubule wall (Fig. 2-3C, 2-3D). Spermatids and spermatozoa began to form in the centre of the lumen in the latter parts of this stage. Development of these cells occurred in columns from the tubule wall that often converged towards the testis lumen (Fig. 2-3D). Testis weight increased dramatically from the growth stage (Table 2-1).

Shedding (V).

Testes in this stage had the lumen filled with spermatozoa, and often a thin layer of spermatocytes remaining along the tubule wall (Fig. 2-3E). The tubule wall was thin and showed reduced staining with eosin (Fig. 2-3E). The testis reached its maximum weight (Table 2-1) and could weigh up to 80.7 mg. Spermatozoa in Leptosynapta clarki have a small circular head about 3.0 µm in diameter and a tail flagellum measuring 35-45 µm in length (Atwood 1974b).

During this stage there was evidence for protandry in some specimens. The lumen was packed with spermatozoa, but along the tubule wall were the very earliest stages of egg formation (Fig. 2-3F).

Post-spawning (I).

After spawning, the testis was divided into areas of nodal swelling containing remnant spermatozoa adjacent to thinner areas lacking sperm. Some spermatozoa were visible in the lumen, and eggs if present were visible along the tubule wall (Fig. 2-3G, 2-3H).

For maturity indices male post-spawning gonads were divided into two categories: (i) Those gonads that were resorbed to leave a small pigmented tubule on the gonad basis. Such gonads were too small and fragile to examine histologically after December 1989 (Table 2-1); (ii) Those gonads completing protandrous sex change. Histologically these gonads are equivalent to the recovery stage of females except for the presence of remnant sperm from the previous reproductive season (Fig. 2-3H). These gonads are described as male Post-spawning (ii) until the release of pentactulae from the

females in April. At this point, it was impossible to differentiate between females who had released pentactulae and males who had undergone protandric sex change.

b. Female.

Recovery (II).

In the recovery stage, the early vitellogenic eggs (17-44 µm) were spread throughout the tubule (Fig. 2-4A) or closely associated with the tubule wall (Fig. 2-4B). In the latter case the oocytes could be elongated because of spatial constraints (Fig. 2-4B). PAS+ material begins to develop in the cytoplasm of oocytes as small as 25 µm in diameter (Fig. 2-4C) and accumulates during the recovery and growth stages. In the latter parts of this stage some larger oocytes (70-90 µm) begin to recruit into the tubule lumen (Fig. 2-4B). Haematoxylin-staining nuclei may be present in the tubule wall as in males.

Growth (III).

Ovaries in this stage show more advanced oocyte development (Fig. 2-4D, 2-4E) and are considerably larger than in the recovery stage (Table 2-1). Larger oocytes (80-110 µm) are present in the lumen surrounded by follicular epithelium with accessory/follicle cells (Fig. 2-4D, 2-4E, 2-4F). There is active accumulation of yolk material (Fig. 2-4D), and oocytes > 90 µm in diameter are strongly PAS+ and a deep magenta colour.

Mature (IV).

In the mature stage larger PAS+ oocytes (120-180 µm) filled the tubule lumen (Fig. 2-4G, 2-4H). The tubule wall was usually thin (Fig. 2-4G, 2-4H) and clear in fresh material. Degenerating oocytes may begin to appear in this stage, though these were more common in the prefertilization stage.

Prefertilization (V).

There was no clear distinction between the mature stage (IV) and the prefertilization stage (V) as the oocytes are not spawned from the ovary. The tubules were dominated by larger oocytes (150-180 µm; fresh material 200-240 µm) surrounded by follicular epithelium and not loose in the tubule (Fig. 2-5A).

Although the ovaries are in their most advanced stage, oocyte proliferation may occur in nests along the tubule wall adjacent to larger oocytes (Fig. 2-5B). This growth of oocytes was not restricted to the basal end adjacent to the gonad basis, but occurred

Figure 2-4. Histological sections of female Leptosynapta clarki in the recovery, growth and mature stages.

A. Recovery stage ovary with developing oocytes. Section stained with haematoxylin/eosin. Bar= 100 µm.

B. Recovery stage ovary with more advanced oocyte development. Note elongated shape of oocytes due to spatial constraints along the ovary wall. Section stained with haematoxylin/eosin. Bar= 50 µm.

C. Recovery stage ovary showing vitellogenesis in small oocytes. Section stained with haematoxylin/PAS. Bar= 100 µm.

D. Growth stage ovary with larger oocytes. There are some degenerating oocytes (d) in this stage. Section stained with haematoxylin/PAS. Bar= 100 µm.

E. Growth stage ovary with bimodal distribution of oocyte size. Small and large oocytes are closely associated in the tubule. Section stained with haematoxylin/eosin. Bar= 50 µm.

F. Growth stage ovary showing follicular epithelium (fe) and accessory cells (arrows) surrounding oocytes. Section stained with haematoxylin/eosin. Bar= 25 µm.

G. Mature stage ovary. Large oocytes fill tubule; thin tubule wall. Section stained with haematoxylin/eosin. Bar= 100 µm.

H. Mature stage ovary stained with haematoxylin/PAS. Bar= 100 µm.

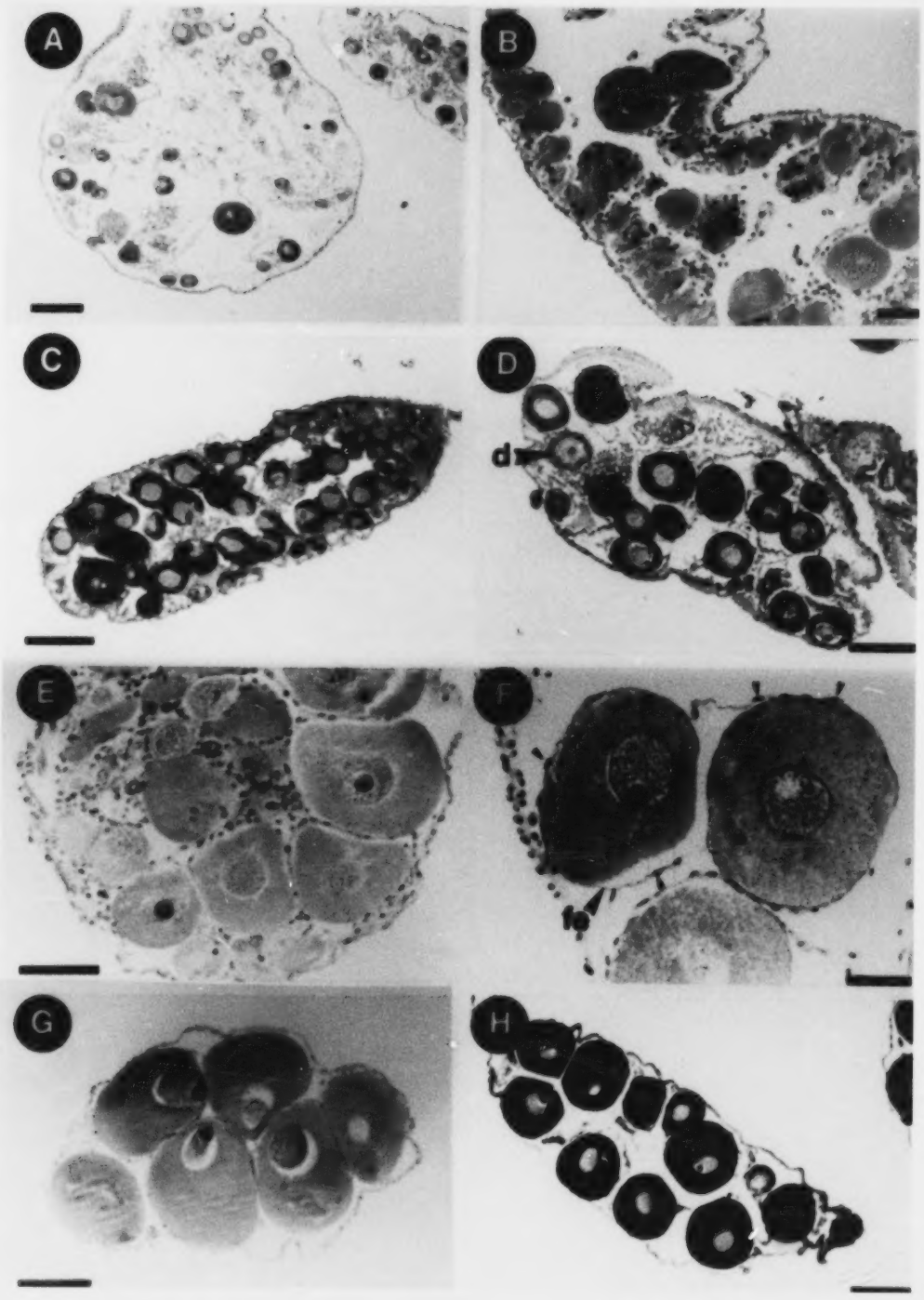
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Figure 2-5. Histological sections of female Leptosynapta clarki in the mature, prefertilization and post-spawning stages.

A. Prefertilization stage ovary with mature oocytes. Section stained with haematoxylin/eosin. Bar= 100 µm.

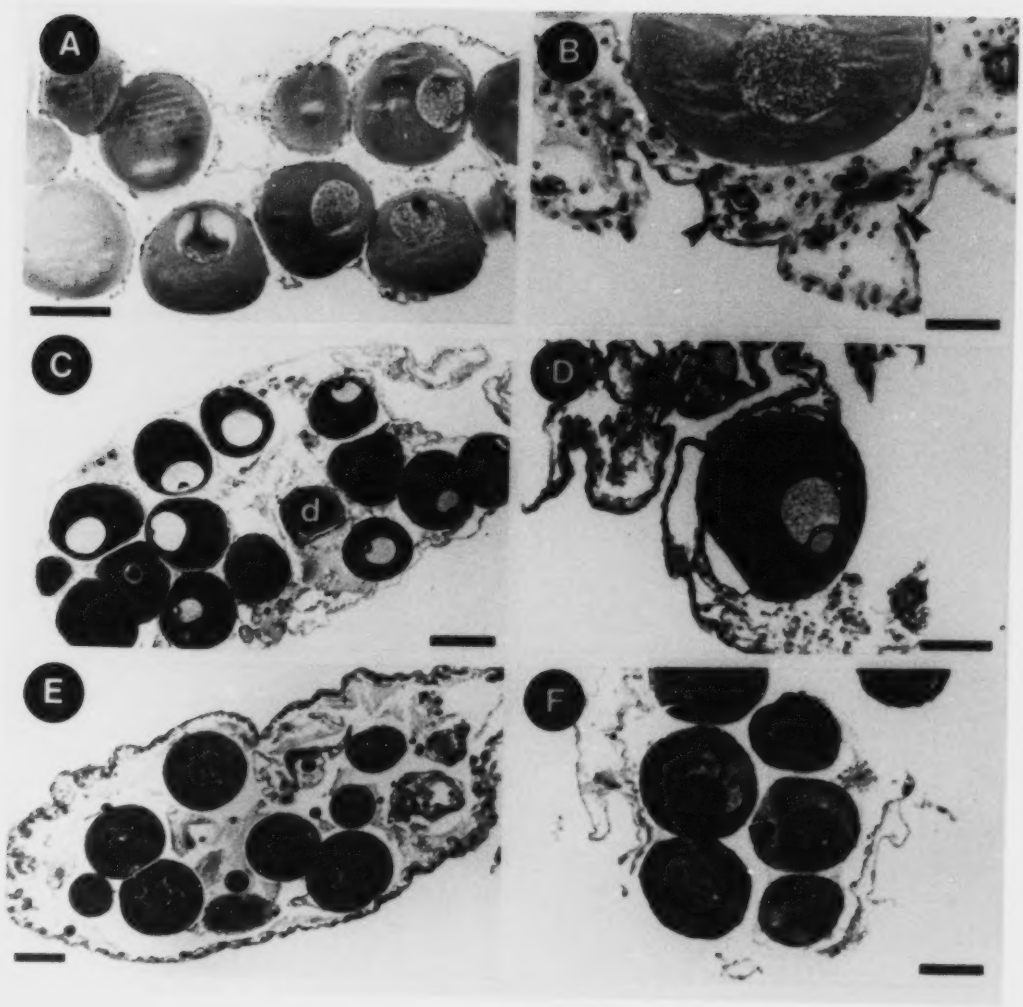
B. Prefertilization stage ovary with a nest of small oocytes (arrows) adjacent to a mature oocyte. Section stained with haematoxylin/eosin. Bar= 25 µm.

C. Prefertilization stage ovary with prominent textural changes in ovarian wall. Note the presence of degenerating oocytes (d) with mature oocytes. Section stained with haematoxylin/PAS. Bar= 100 µm.

D. Prefertilization stage ovary with large and small oocytes associated with ovary wall. Well developed texture is seen in the ovarian wall. Section stained with haematoxylin/PAS. Bar= 50 µm.

E. Post-spawning stage ovary with degenerating oocytes and textured ovarian wall. Section stained with haematoxylin/PAS. Bar= 100 µm.

F. Post-spawning stage ovary with degenerating oocytes and textured ovarian wall. Resorption involves the separation of the cytoplasm of the oocytes into discrete droplets. Section stained with haematoxylin/PAS. Bar= 100 µm.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along the entire length of the tubule including the distal tip. Many degenerating oocytes began to appear in this stage (Fig. 2-5C), and could comprise the majority of oocytes in the tubule.

During the prefertilization stage the tubule wall changed its form by increasing its thickness and changing its texture (Fig. 2-5C, 2-5D). The development of this texture made the ovarian wall opaque in contrast to the thin clear wall seen in the mature stage (Chapter 3), and there was usually an associated colour change from yellow to pink. Changes in the tubule wall during the prefertilization stage are examined using TEM in Chapter 3.

Post-spawning (I).

As the oocytes in Leptosynapta clarki are not released during reproduction, it should be noted that the post-spawning stage in females occurs during the period of spawning in males. Ovaries in the post-spawning stage are at their maximum weight (Table 2-1) and may fill the majority of the coelomic space.

This stage is subdivided into two parts, dependent on whether fertilization was successful:

(i) Fertilization successful; embryonic development in the tubules.

Embryonic development in Leptosynapta clarki from Bamfield is similar to that described from False Bay (Everingham 1961). Hatching occurs after the prepentactula stage (Fig. 2-6A) to form a 5-tentacled pentactula (Fig. 2-6B) which is able to move freely in the ovarian tubule. Development to the pentactula stage in filtered sea water occurred in ca. 10 days to 2 weeks. The majority of the brooding period, therefore, is spent as a pentactula. Secondary tentacles appear between the five primary tentacles while the pentactula is in the ovarian tubule (Fig. 2-6C). The most advanced pentactula observed in the tubules had eight tentacles (Fig. 2-6D). Many juveniles are released when they have 8 tentacles, though rare specimens with 9, 10 or 12 tentacles were observed in the ovarian tubule by Everingham (1961).

Within an ovary only a portion of the eggs are fertilized and undergo embryonic development. The remaining unfertilized eggs within the ovary degenerate (Fig. 2-5E, 2-5F). During this degeneration the cytoplasm breaks into many discrete droplets (Fig. 2-5F) which become free in the tubule after the breakdown of the egg membrane (Fig. 2-6F). The droplets are predominantly found along the tubule wall adjacent to the textured ovarian wall (Fig. 2-6C).

Droplets formed from resorbing unfertilized oocytes are used as food by the pentactulae and are visible in the intestine (Fig. 2-6E, 2-6F). Later in the brooding

Figure 2-6. Histological sections of female Leptosynapta clarki in the post-spawning stage with embryonic development. All sections stained with haematoxylin/eosin.

A. Prepentactulae in the ovarian tubules. Note that the embryos are yet to hatch and that there are droplets from the breakdown of unfertilized eggs in the tubule lumen. Bar= 100 µm.

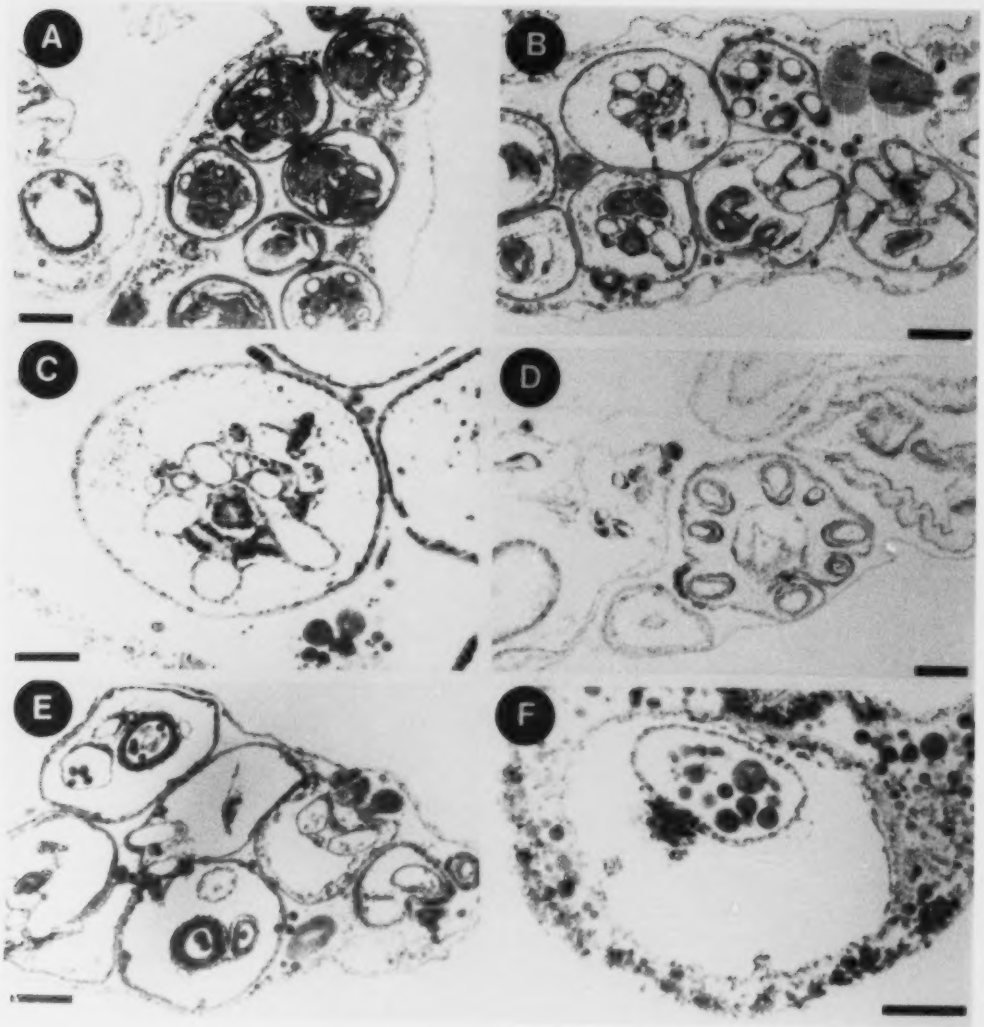
B. Pentactulae with five tentacles in the ovarian tubule. The ovarian wall is still textured and there are some resorbing eggs. Bar= 100 µm.

C. Pentactula at a later stage with secondary tentacles appearing between the five primary tentacles. Note that the droplets from the breakdown of resorbing eggs are concentrated along the ovarian wall. Bar= 50 µm.

D. Eight-tentacled pentactula in ovarian tubule. Note the tubule wall has decreased in thickness. Bar= 100 µm.

E. Pentactulae with droplets from resorbing oocytes in the intestine. Ovarian wall still retains some textured appearance. Bar= 100 µm.

F. Closeup of pentactula showing droplets from resorbing oocytes in intestine. Note how tubule is filled with resorbing droplets. Bar= 100 µm.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period there are no discrete droplets visible in the tubules and the pentactulae feed on the fluid itself (Everingham 1961, present study).

In the latter part of the brooding period the textured appearance of the tubule wall observed in the prefertilization stage is lost. The tubule wall becomes very thin (Fig. 2-6D). Small oocytes begin to form along the tubule wall in the same region as the pentactulae (Fig. 2-7A, 2-7B). It was confirmed using TEM that these are in fact oocytes (Fig. 2-7C).

At the end of the brooding period pentactulae are released from the female by autotomy or rupture of the body wall and are seen as a discrete recruitment pulse in the sediment in early May (Chapter 6).

(ii) No fertilization.

In some ovaries no fertilization occurs. The unfertilized eggs are free in the ovarian lumen (Fig. 2-5E, 2-5F) and are resorbed as described above. In fresh material females were noted with resorption at the distal end and new egg growth at the basal end adjacent to the gonad basis (Fig. 2-2D).

3. Reproductive cycle.

a. Gonad Indices.

The gonad index proved to be a reliable indicator of gonad development in Leptosynapta clarki. Although similar patterns were observed in the gonad indices at both sites (Fig. 2-8) there were significant differences in the value of GI between sites (Table 2-2). Male Leptosynapta clarki generally had a higher mean and maximum GI at Site 2 (Table 2-3). In females, values of the mean gonad index were more variable because of differing degrees of fertilization between individuals.

From a low mean value of 0.1% in April or May the male GI increased throughout the summer months, reaching a maximum in late November/early December in both 1989 and 1990 (Fig. 2-8). Spawning was generally synchronous as shown by the decreased male GI in December and January at both sites (Fig. 2-8). Resorption of the testes following spawning reduced the GI to its summer minimum (Fig. 2-8).

Trends in the male gonad index with time were followed only in adult sea cucumbers. Year 1 males, released from the mother in the current year, are reproductively active and invest a similar proportion of body weight in reproduction. In November 1990, dissections of Year 1 males (31-116 mg total weight) showed gonad indices as a percent of drained weight from 1.58-11% (Mean ± SD: 5.86 ± 2.99%, N= 7). The mean gonad index for older males at Site 1 at this time was 5.49 ± 2.09% (N= 15).

Figure 2-7. Evidence for oocyte development in the same tubule as pentactulae development.

A. Histological section of pentactula (p) with small oocytes adjacent to the tubule wall. Section stained with haematoxylin/PAS. Bar= 100 µm.

B. Histological section of posterior portion of pentactula (p) and developing oocyte. Section stained with haematoxylin/PAS. Bar= 50 µm.

C. TEM of oocyte from tubule with pentactulae in February 1990. Bar= 5 µm.

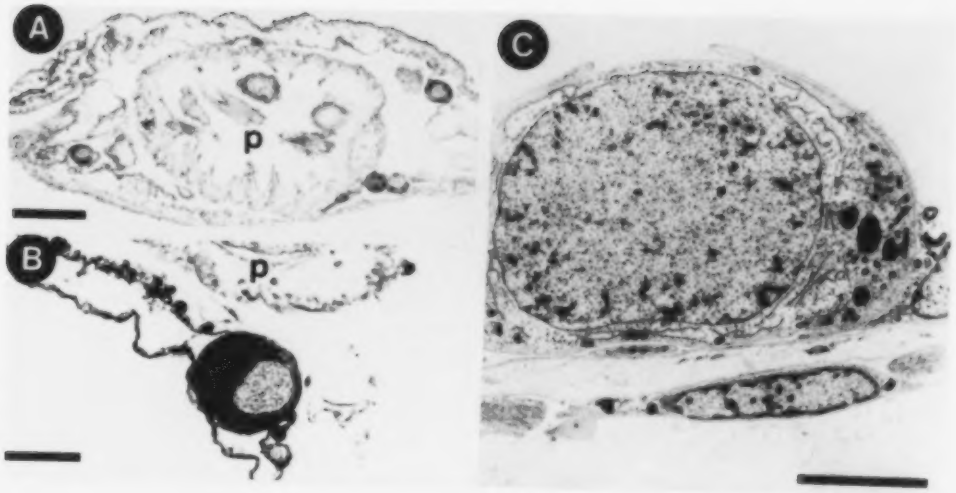

A
p
B
p
C

Figure 2-8. Mean gonad index for adult male (circles) and female (squares) Leptosynapta clarki collected from Grappler Inlet. A. Site 1. B. Site 2. Error bars represent the standard error of the mean.

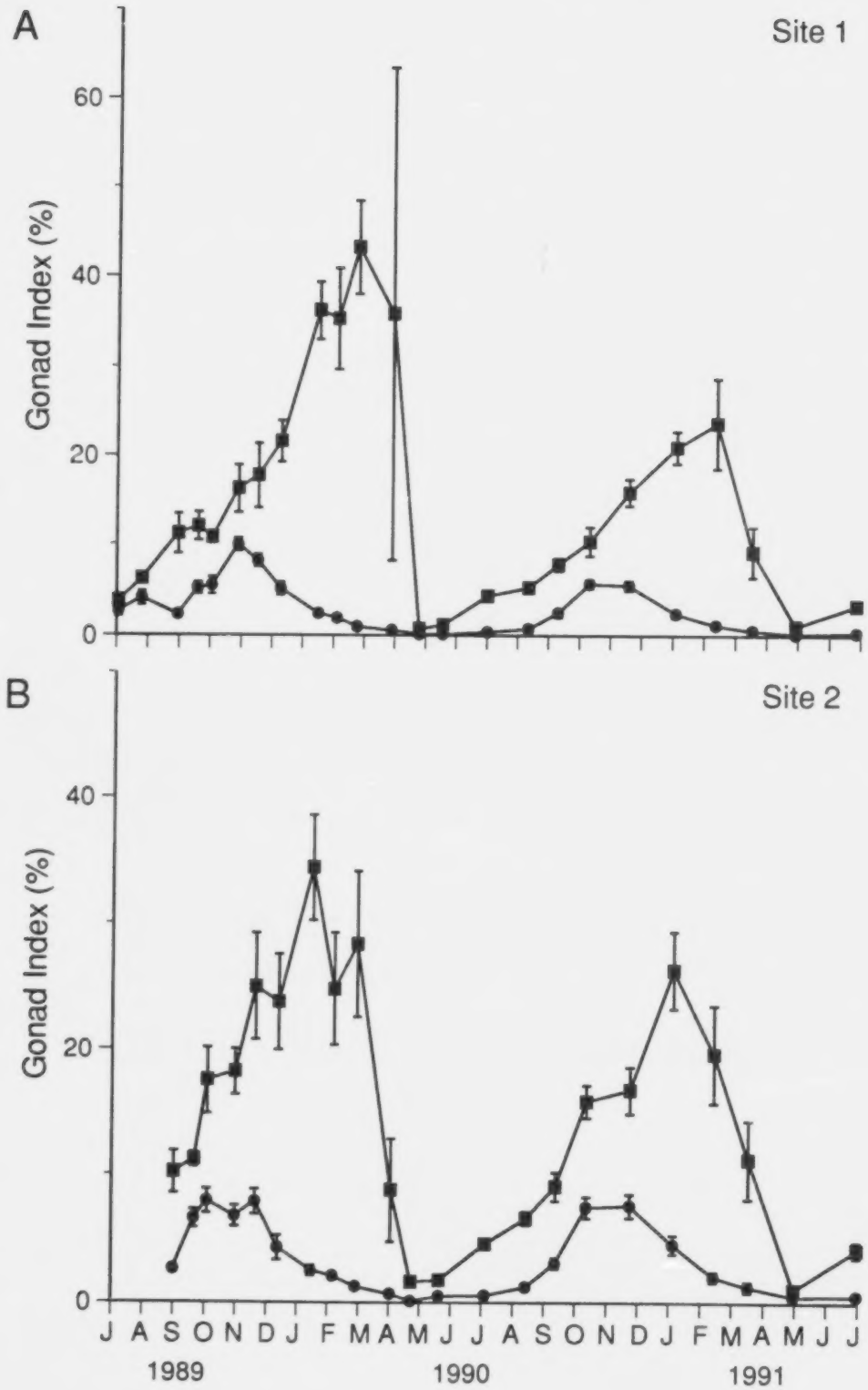

A
Site 1
Gonad Index (%)
60
40
20
0
B
Site 2
Gonad Index (%)
40
20
0
J A S O N D J F M A M J J A S O N D J F M A M J J
1989
1990
1991

Table 2-2. Analysis of variance testing gonad index (arcsin-transformed) between sites from August 1989 to July 1991. Analysis is a crossed design with sex and sample date (1-22) as main factors, and blocked by site.

Source	df	SS	MS	F	p
Site	1	0.056	0.056	8.74	0.0032
Sex	1	12.077	12.077	1896.36	0.0001
Sample	21	13.301	0.633	99.45	0.0001
Sample*Sex	21	4.891	0.233	36.57	0.0001
Error	1270	8.088	0.006		
Total	1314	35.346			

Table 2-3. Maximum gonad indices for male and female Leptosynapta clarki at Sites 1 and 2, Grappler Inlet. Gonad index calculated as a percent of drained weight.

Maximum Gonad Index (%)	1989	1990
Site 1:		
Male	15.64	9.71
Female prefertilization	27.29	26.80
Female with pentactulae	63.40	46.26
Site 2:		
Male	20.44	15.26
Female prefertilization	29.70	20.65
Female with pentactulae	56.95	47.72

The female gonad index showed a similar pattern to the males, rising through the summer and early autumn (July-Oct.; Fig. 2-8) to reach a maximum before fertilization of over 20% (Table 2-3). The mean gonad index in female Leptosynapta clarki was always higher than males (Fig. 2-8, Table 2-3), and continued to rise after spawning because of the weight of pentactulae within the ovaries (Fig. 2-8). The standard error of the mean gonad index was maximal during the brooding period as the index included a range of reproductive conditions: no fertilization/resorption, a few or hundreds of pentactulae (Fig. 2-8). A rapid decline in the index was seen in the spring (April, May) when the pentactulae were released from the female (Fig. 2-8). The mean minimum gonad index observed in females after pentactulae release was 0.70% and 1.00% during April or May at Site 1 and 2 respectively (Fig. 2-8). The higher minimum value observed in females at this time is partly the result of including protandric males with advanced oocyte development in the calculation of mean female GI.

Differences in the mean female gonad index during the brooding periods of 1989 and 1990 were primarily due to the lower number of females brooding pentactulae in 1990 (Fig. 2-9). Lower values for the maximum gonad index in both sexes prior to spawning in 1990 (Table 2-3) and poor fertilization success during the spawning season resulted in fewer females brooding pentactulae in 1990 (Fig. 2-9) and thus decreased values for the mean gonad index (Fig. 2-8).

b. Gametogenic cycle.

The relative frequencies of the maturity stages for male and female Leptosynapta clarki in Grappler Inlet are shown in Figure 2-10 from July 1989 to August 1990. In the summer months the gonads in both sexes begin to mature (Fig. 2-10, 2-11) and increase in size. Male recovery stage gonads appear from July-September and are followed by the rapid maturing of the testes to reach a shedding state by early November (Fig. 2-10, 2-11). Shedding stage gonads were present from early October to mid December (Fig. 2-10). In addition, the population includes reproductively active male sea cucumbers that were released from the female in May of the current year (Chapter 6). These males were excluded from gonad index calculations and from histology due to their small size (mean total weight 53.4 mg; Chapter 6).

Post-spawning (i) males are present in November -December during the period of spawning (Fig. 2-10) resulting in a drop in the maturity index (Fig. 2-11). Increasing numbers of Post-spawning (ii), or transitional males, are found in the late winter and spring (Jan.-April, Fig. 2-10) as these males had large enough gonads for histology.

Figure 2-9. Percent of brooding female Leptosynapta clarki in each sample from July 1989-1991. Sites 1 and 2 combined. Females with germinal vesicle breakdown (GVBD) defined as brooding; females with no living pentactulae in ovary defined as a resorbing gonad (i.e. not brooding). Number adjacent to each point represents the number of sea cucumbers used to calculate percentage.

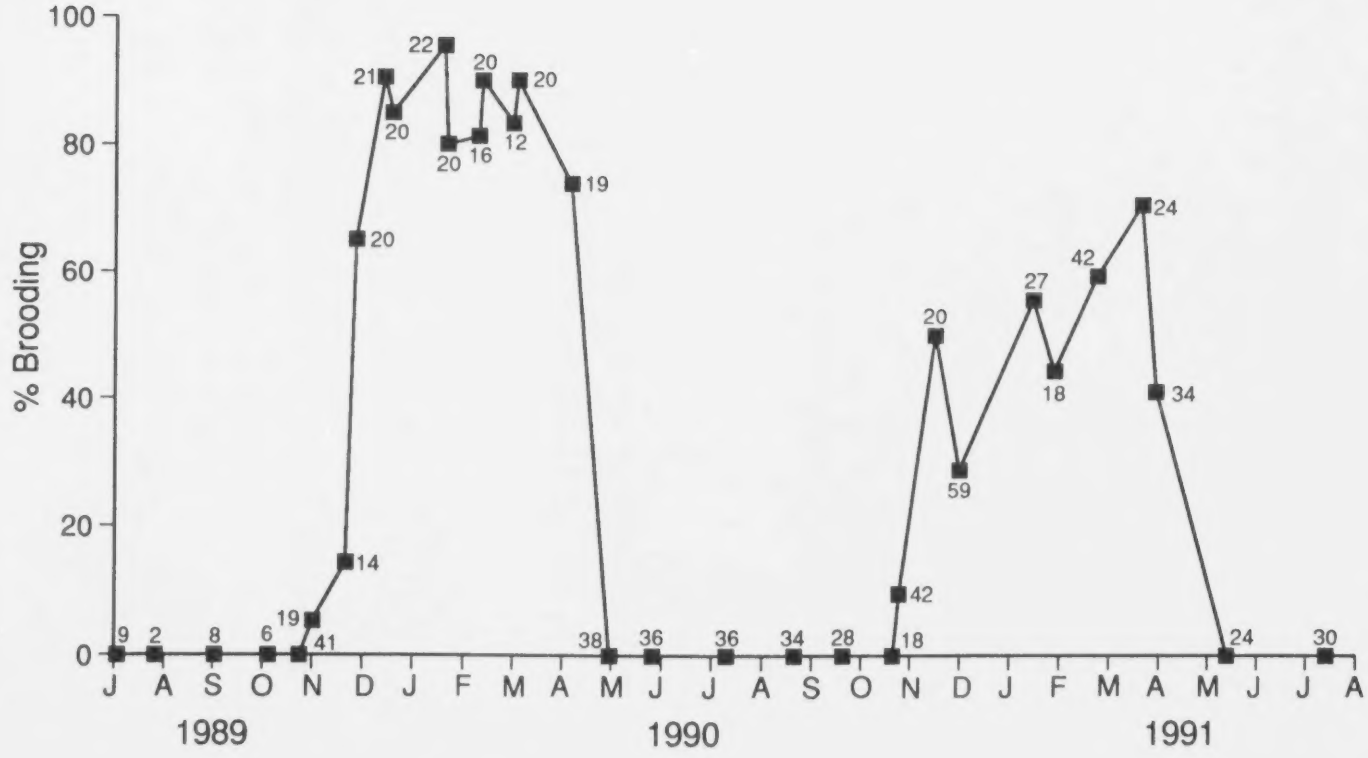

% Brooding
100
80
60
40
20
0
J A S O N D J F M A M J J A S O N D J F M A M J J A
1989
1990
1991
9 2 8 6 19 41 14 20 21 20 22 20 20 16 20 12 20 19 38 36 36 34 28 18 42 20 59 27 18 42 24 34 24 30

Figure 2-10. Maturity charts for male and female Leptosynapta clarki. Chart shows percent of sea cucumbers in each maturity stage. Site 1 and 2 combined. Maturity stages are defined in text. Numbers above each bar refer to the number of sea cucumbers on which percentage is based.

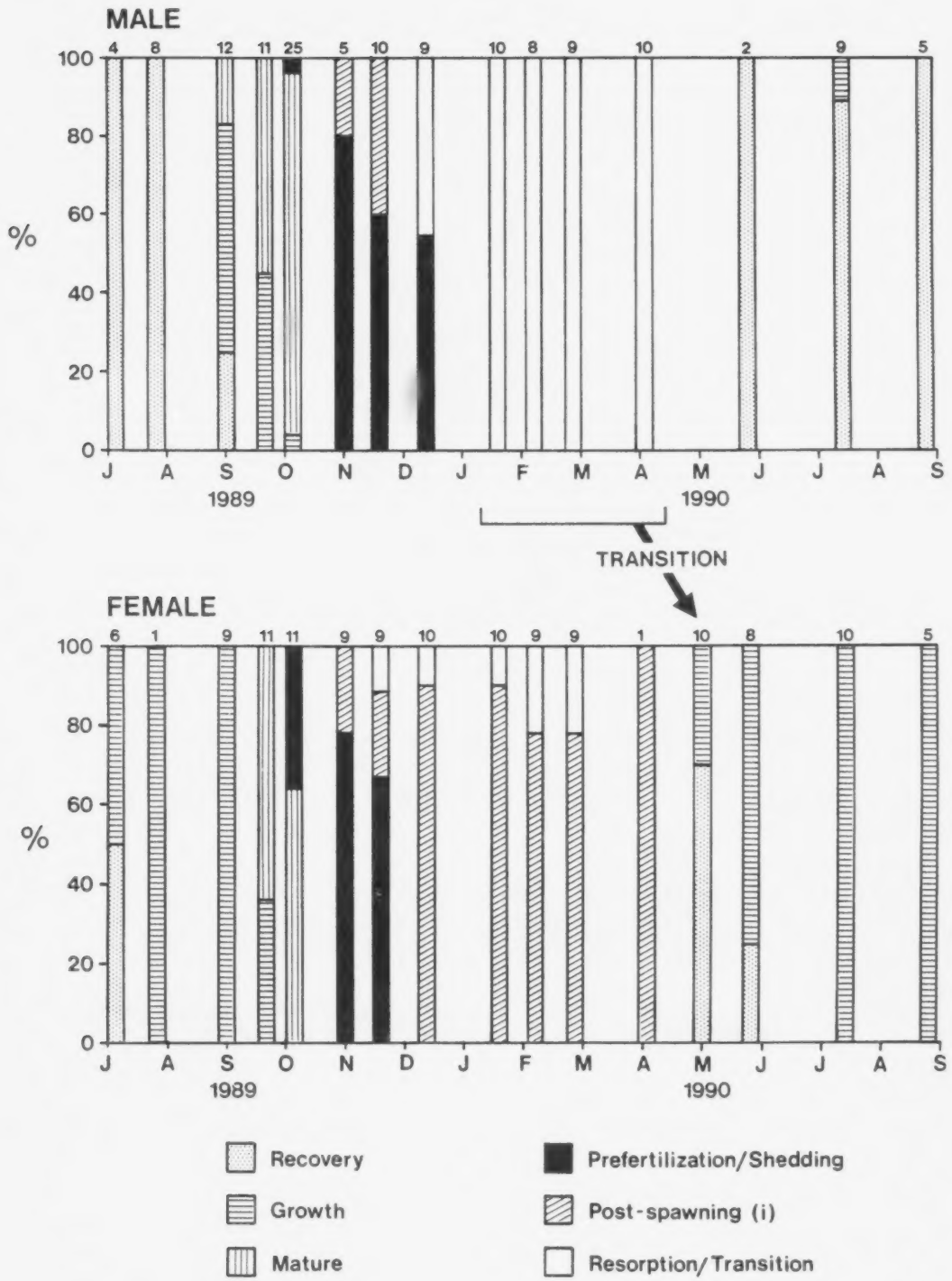
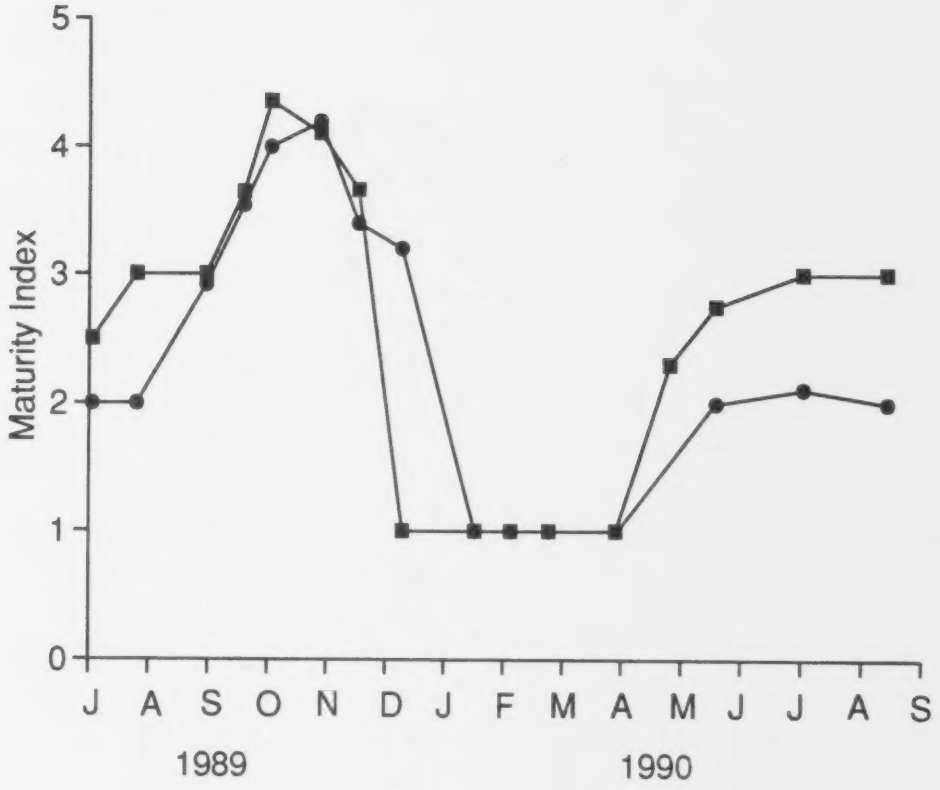

MALE
4 8 12 11 25 5 10 9 10 8 9 10 2 9 5
100
80
60
40
20
0
%
J A S O N D J F M A M J J A S
1989
1990
TRANSITION
FEMALE
6 1 9 11 11 9 9 10 10 9 9 1 10 8 10 5
100
80
60
40
20
0
%
J A S O N D J F M A M J J A S
1989
1990
Recovery
Growth
Mature
Prefertilization/Shedding
Post-spawning (i)
Resorption/Transition

Figure 2-11. Maturity indices for male (circles) and female (squares) Leptosynapta clarki. Sites 1 and 2 combined. Post-spawned (i) and (ii) are combined. Maturity indices calculated from Yoshida (1952).


Maturity Index
5
4
3
2
1
0
J A S O N D J F M A M J J A S
1989
1990

Female Leptosynapta clarki also show a rapid period of maturation (Fig. 2-10, 2-11). Recovery and growth stages are present in the late summer and early autumn, and form the prefertilization stage coincident with the shedding stage in males (Fig. 2-10). Fertilization is first evident in early November; brooding continues until April or May the following year (Fig. 2-9, Fig. 2-10). Unfertilized ovaries are also present throughout the brooding period (Fig. 2-10), and by February or March consist of little recognisable egg material. After the breaking of the ovary in pentactulae release, the basal end of the ovary is defined in either the recovery or growth stage (Fig. 2-10).

c. Spawning.

Male Leptosynapta clarki were never observed to spawn in either the field or the laboratory. Observations on night time low tides during the spawning period revealed only feeding movements and no aggregative behaviour as observed in Leptosynapta clarki at False Bay (Everingham 1961, McEuen 1988). A few males in the laboratory shed a small amount of sperm in response to handling, and sperm could be activated in the ripe testis by exposing the coelom to sea water.

During the spawning season, a black pigmentation was observed around the tentacles and gonopore of a wide size range of males (112-1357 mg total weight, N= 31), and in two female sea cucumbers. This pigmentation appears to be related to spawning as males were not observed to have this pigmentation at other times of the year and males with totally spawned out gonads had light or no pigmentation. The importance of this pigmentation pattern to spawning in Leptosynapta clarki is unknown.

D. Discussion.

The morphology and histology of the ovary in Leptosynapta clarki resembles that described for non-brooding holothurians. For example, the oocytes of Leptosynapta clarki are surrounded by a follicular epithelium with follicle cells (Everingham 1961, present study) as in other holothurians (Apodida: Menker 1970, Green 1978; Aspidochirotida: Smiley and Cloney 1985, Smiley 1988, Tyler et al. 1992; Dendrochirotida: Tyler and Gage 1983, Costelloe 1985 Hamel et al. 1993; Elasipodida: Tyler et al. 1985b, Tyler and Billett 1987; Molpadida: Tyler et al. 1987).

However, in two respects oocyte formation differs from other sea cucumbers. Firstly, the retention of the pentactulae in the ovary means that the usual pattern of resorption of the spent ovarian tubules after spawning (Hyman 1955, Smiley 1988) does not occur. Instead, oocytes for the next year's reproductive period are produced within

the same ovarian tubule giving overlapping generations of oocytes. Secondly, there are two periods of oocyte growth, dependent on the sex of the sea cucumber in the previous reproductive season.

Overlapping generations of oocytes, where immature and mature oocytes are found in the same ovarian tubule, have been observed in many holothurians (Tanaka, 1958, Pearse 1968, Menker 1970, Green 1978, Engstrom 1980, Estabrooks 1984, Costelloe 1985, Ong Che and Gomez 1985, Tyler et al. 1987, Tyler and Billett 1987, Gutt et al. 1992, Sewell 1992, Tyler et al. 1992, Hamel et al. 1993). In species where the spent ovarian tubule is resorbed (Choe 1963, Harriott 1982, Cameron and Fankboner 1986, Sewell 1992) these immature oocytes are resorbed with the rest of the tubule. However, in Leptosynapta clarki as the ovarian tubule is not resorbed, the smallest cohort of oocytes remains and grows in the ovarian tubule for next year's reproductive period. Similar observations have been made in the ophiuroid Acrocnida brachiata (Bourgoin and Guillou 1990) and the sea cucumber Aslia lefevrei (Costelloe 1985) where a new generation of oocytes was present before the gonads had completely emptied.

Individuals of Leptosynapta clarki have immature oocytes in the current ovarian tubule (with pentactulae or resorbing oocytes), or sex-changing testes. This means that reproduction for the next year continues in the previously fecund tubule. This observation is important in assessing the relevance of the "tubule recruitment model" (Smiley 1988) for the ovarian cycle in Leptosynapta clarki. Central to the "tubule recruitment model" is the progressive recruitment of tubules on the gonad basis (Smiley 1988). The fact that there is only a single gonad tubule attached to the gonad basis on each side of the dorsal mesentery, and the differentiation of oocytes with either pentactulae or spermatozoa strongly suggest that the "tubule recruitment model" (Smiley 1988) is not applicable in this species.

The "tubule recruitment model" as proposed by Smiley (1988) may be relevant to only a subset of holothurian species (see Smiley et al. 1991, Tuwo and Conand 1992) and only in some populations. Sewell (1992), for example, found that this model might be applicable to Stichopus mollis in the South Island of New Zealand but not in northern populations. Hamel et al. (1993) described two sizes of gonadal tubules in Psolus fabricii but found a markedly different pattern of tubule and gamete production in comparison to Parastichopus californicus in which the "tubule recruitment model" is based (Smiley 1988). Additionally, the model may not be applicable in small short-lived holothurians (Smiley et al. 1991), in sex-changing species (present study) or species such as deep sea holothurians with continuous reproduction (Tyler and Gage 1983, Tyler et al. 1985a,

1985b, Tyler et al. 1987, Tyler et al. 1992). The generality of this model to reproduction in holothurians, therefore, requires further research.

Two periods of oocyte proliferation occur in Leptosynapta clarki dependent on the sex in the previous reproductive season. Brooding females initiate oocyte growth in the latter part of the brooding period, primarily at the basal end adjacent to the gonad basis, but also in the distal area where pentactulae are being brooded (Fig 2-2C). Active oocyte proliferation does not, however, occur until after pentactulae are released in April or May giving recovery stage gonads in May and June (Fig. 2-10). In contrast, sex-changing males may begin oogenesis in November when the testis lumen is filled with spermatozoa. The majority of transitional males have active oocyte proliferation from December to May, and have reached the female growth stage by the time brooding females have released the pentactulae (Fig. 2-10).

The time to produce mature oocytes, therefore, differs between the sexes. The appearance of oocytes in the testes of protandric males in November and December suggests that 11-12 months are taken for oocyte growth. In contrast, oocyte growth in females is completed in the 6 months after pentactulae release (May-November). A similar difference in the time required for gametogenic growth with season has been observed in Leptosynapta tenuis (Green 1978). Gametogenic growth completed in 5 months in the winter and early spring could occur in as little as a month in summer or autumn (Green 1978).

Male Leptosynapta clarki show an annual reproductive cycle producing spermatozoa each year for spawning in November and December. The process of testis development in Leptosynapta clarki is similar to that described in other holothurians. The longitudinal folds, or convolutions, in the inner epithelium of the testes are seen in several other species (Théel 1901, Tanaka 1958, Engstrom 1980, Costelloe 1985, Tyler et al. 1985a, 1985b, Cameron and Fankboner 1986, Tyler and Billett 1987, Tyler et al. 1987, Sewell 1992, Tuwo and Conand 1992). These folds may have two purposes. Firstly, the folds increase the surface area of the germinal epithelium (Cameron and Fankboner 1986) allowing greater proliferation of spermatogonia. Secondly, the distal side of these longitudinal folds are filled with haemal fluid (Smiley et al. 1991) and may provide nutrition for the developing spermatocytes and spermatogonia.

In contrast to other holothurians (Smiley et al. 1991) some germ line cells must remain in the inner epithelium of the Leptosynapta clarki testes after the proliferative phase. In sex-changing individuals these germ line cells are activated to form previtellogenic eggs. Other protandric species such as the limpet Patella oculus also instigate oocyte growth during the period when the testes have mature spermatozoa

(Branch 1974). This species can be shown histologically to develop oocytes which enlarge and replace the remnants of sperm (Branch 1974) as in Leptosynapta clarki (Fig. 2-3G, 2-3H, Chapter 4).

Shedding stage testes with large numbers of spermatozoa were found only in the period from early October to mid December (Fig. 2-10) when spawning occurred (Fig. 2-8). There are few observations of spawning in apodid sea cucumbers. Both Labidoplax buskii (Nyholm 1951) and Leptosynapta inhaerens (Runnström 1927, in Hyman 1955) emerge part way from their burrows and release sperm into the water column accompanied by swaying movements. In contrast, Leptosynapta clarki are reported to spawn at low tide in False Bay, San Juan Island (Everingham 1961, McEuen 1988). Groups of 2 to 4 individuals were found by these authors lying close together or intertwined during the spawning period. However, as neither Everingham (1961) nor McEuen (1988) observed spawning or found spermatozoa in the depressions formed by these aggregations, it is unknown whether these groups are in fact formed by spawning individuals.

While fertilization in Leptosynapta clarki must occur internally, the method by which sperm enters the ovary is as yet unknown (Everingham 1961, McEuen 1987, Hess et al. 1988). Anderson (1966) suggested that sperm might enter the coelom of Leptosynapta clarki through anal pores. As this would require sperm to pass through the ovarian wall prior to fertilization it is more likely that sperm enters through the gonopore. The fact that embryonic development in the ovary of Leptosynapta clarki occurs synchronously (Everingham 1961, unpub. obs.) suggests that sperm are entering at one point in time.

An unusual observation in Leptosynapta clarki is that exposing the testis to sea water activates spermatozoa within the tubule. Spermatozoa in echinoderms are usually non-motile during storage in the testis although high partial pressures of oxygen can induce spermatozoan motility in undiluted semen (Chia and Bickell 1983). Opening the coelom and exposing the testes to higher oxygen concentrations in the surrounding sea water might therefore activate the sperm. As Leptosynapta clarki is a protandric hermaphrodite, there might be the potential for self-fertilization during the transitional phase. This is, however, unlikely as mature sperm and oocytes are not present at the same time, and the sperm would have to survive and fertilize the oocytes some eight months later (Everingham 1961). In addition, electrophoretic studies of L. clarki on San Juan Island show no evidence for self-fertilization (Hess et al. 1988). Studies on spawning and fertilization in Leptosynapta clarki are essential to determine if self-fertilization is possible.

Intraovarian brooding in Leptosynapta clarki provides two major changes in the reproductive cycle in comparison to other holothurians. Firstly, the oocytes are not spawned from the ovary, and secondly, the mother provides space and nutrition within the ovary for her developing pentactulae. Nutrition for pentactulae in the ovary is provided by the mother primarily from the degeneration of unfertilized eggs in L. clarki and perhaps in Taeniogyrus contortus (Ludwig 1898). These unfertilized eggs break into droplets, "yolky spheres" (Everingham 1961) or "granules" (McEuen 1986) that are seen in the intestine of developing pentactulae (Everingham 1961, McEuen 1986, present study). However, during the brooding period some pentactulae die from undetermined causes (Chapter 5). The body tissues of these dead pentactulae are resorbed in the ovarian tubule and may provide further nutrition for the remaining pentactulae (Chapter 5). Additional nutrients for pentactulae growth might also be derived by diffusion from the haemal system or from the perivisceral coelom (Chapter 3).

Preliminary observations on coelomic brooding holothurians also suggest that nutrition is provided by fluid in the area of brooding. Materia et al. (1991) found that embryos of Staurothyone inconspicua are free in the coelom or attached to the ends of the gonad tubules. Where embryos are attached to the gonads, Materia et al. (1991) suggest that nutrition is derived directly from the ovary; presumably embryos free in the coelom feed on coelomic fluid. In the coelomic brooders Neoamphicyclus lividus and Synaptula hydriformis the embryos ingest coelomic fluid (Hickman 1978, Frick et al. 1992). In the latter species the coleomic fluid contains many coelomocytes and may contain yolk granules or degraded eggs (Frick et al. 1992).

The breakdown products from unfertilized eggs might also be important for the mother's nutrition during the period of brooding. Droplets of yolk in Leptosynapta clarki tended to concentrate along the ovarian wall and might be incorporated into the haemal system, and hence be used by the mother for her own nutrition or for gametogenic growth. This material must be used by the female within a short period of time as species of the genus Leptosynapta do not appear to have a distinct storage organ (Féral 1985). Additional nutrition for the pentactulae may be provided from normal feeding activities or uptake of dissolved amino acids through the thin body wall (Féral 1985).

Pentactulae of Leptosynapta clarki can survive and grow in filtered sea water 3-4 months earlier than they are naturally released (unpub. obs.), as in the coelomic brooding Pachythyone rubra (Chaffee unpub. obs., in Smiley et al. 1991). These observations suggest that pentactulae are not obligately dependent on the mother's ability to provide food and protection, but may gain some advantage from being released at a larger size and more advanced state of maturity (Smiley et al. 1991). Alternatively, the release of

young may be associated with an increase in food availability for the mother or juveniles. The externally brooding Cucumaria lubrica releases the young just prior to the spring phytoplankton bloom and at the point where feeding is resumed in C. lubrica adults (Engstrom 1982). In Leptosynapta clarki the pentactulae are released during the spring months when the mudflats are covered with extensive Enteromorpha spp. mats and abundant diatoms (pers. obs.). This seasonal increase in food may fuel rapid juvenile growth (Chapter 6) and oogenesis in post-brooding females.

This study has described gametogenesis in the brooding sea cucumber Leptosynapta clarki. Although the embryos are retained in the ovary for development, the process of gametogenesis is similar to that in non-brooding holothurians. However, as in many other holothurians the spawning and fertilization biology of L. clarki is unknown. In addition, while protandry is a feature of the reproductive cycle we do not know how sex is determined, whether all individuals change sex (Chapter 4), or how long individual L. clarki live. Further research on this, and other apodid sea cucumbers, may answer some of these questions.

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CHAPTER 3

Changes in ovarian wall structure of the intraovarian brooding Leptosynapta clarki during the reproductive cycle.

A. Introduction.

Viviparity, where eggs are fertilized internally and retained in the maternal reproductive system for development (Komatsu et al. 1990, Wourms and Callard 1992), is an unusual and specialised form of reproduction in marine invertebrates. Viviparity offers an interesting problem to reproduction because the gonad has two functions: firstly in the production of gametes, and then, if fertilization is successful, as the structure in which the embryos are brooded (Chia and Walker 1991).

Within the Phylum Echinodermata there are 13 known species that brood their young in the ovary (Chapter 1). In most species information on viviparity is restricted to basic descriptions of the embryology of development and there is limited information on details of ovary structure during reproduction.

During my study of the reproductive biology of the intraovarian brooding sea cucumber Leptosynapta clarki changes were noted in the ovarian wall prior to fertilization (Chapter 2). In this chapter I present information on the structural changes in the ovarian wall using light microscopy and electron microscopy (EM). The results suggest that the ovarian wall is a dynamic structure and its form is related to different phases of reproduction.

B. Materials and Methods.

Leptosynapta clarki were collected from the mid-intertidal mudflats of Grappler Inlet, Bamfield, British Columbia (48°50'N, 125°06'42"W) during February and December 1990, and August 1991. The two ovarian tubules were dissected from each female and fixed for transmission electron microscopy (TEM). Tissues were fixed for 1 hour in 2.5% glutaraldehyde buffered with Millonig's phosphate buffer (pH= 7.6) made isotonic to sea water with NaCl (Dunlap 1966). The ovaries were then rinsed in 1.25% $NaHCO_3$ (pH= 7.2) for 15 minutes before secondary fixation for 1 hour in a 2% osmium tetroxide solution buffered with 1.25% $NaHCO_3$ (pH= 7.2). The tissues were then dehydrated in an ascending ethanol series (30%, 50%, 70% etc.) and embedded in Epon resin (Polysciences). Sections for TEM were stained for 10 minutes in alcoholic uranyl

acetate, followed by 10 minutes in lead citrate and then observed with a Phillips E.M. 201 electron microscope.

Photographs of fresh material were taken immediately after dissection from the female. The ovarian tubules were placed on a glass slide (38 x 75 mm) with a small amount of sea water, temporarily mounted with a coverslip (35 x 50 x 0.02 mm), and photographed with a Leitz compound microscope.

Histological sections were obtained during a detailed study of reproduction in Leptosynapta clarki (Chapter 2). Gonads were preserved in Bouin's fixative, stored in 70% ethanol and embedded in Paraplast. Sections were cut at 7 µm, stained in haematoxylin and eosin, and photographed on a Zeiss photomicroscope.

C. Results.

1. General observations.

The ovary of Leptosynapta clarki is located in the anterior coelom and consists of two tubules, one to each side of the dorsal mesentery as in other synaptids (Hyman 1955). Each tubule is attached at the basal end to a small swelling, the gonad basis, that connects the two tubules through the mesentery to a single gonoduct (Chapter 2). Short lobes branch off the main tubule only along the side furthest from the dorsal mesentery i.e. towards the body wall (Chapter 2).

Ovary growth occurs primarily after release of the brooded pentactulae in April or May (Chapter 2). The oocytes grow and mature within the tubules and are mature from late September to early October (Chapter 2). During late oogenesis fresh ovarieshave a thin clear wall and appear yellow in colour due to reflection of light from the yolk-rich eggs (Fig. 3-1A). In histological sections the ovarian wall is a flattened layer (Fig. 3-1B). This is the primary form of the ovarian wall (Stage 1).

After the majority of the oocytes reached mature size, the ovaries were defined in the prefertilization stage (Chapter 2). The tubules were filled with large oocytes that were surrounded by follicular epithelium and not free in the gonad tubule (Chapter 2). During this stage the tubule wall changed in texture and increased in thickness (Fig. 3-1C). The development of this texture made the ovarian wall opaque, in contrast to the clear appearance of the less developed ovary (Fig. 3-1A), and there was usually an associated colour change from yellow to pink (Stage 2). Histologically this resulted in a thickened tubule wall with extensions from the inner surface of the wall towards the ovarian lumen (Fig. 3-1D). The ovarian wall retained this form during most of the brooding period.

Figure 3-1. Ovarian wall structure in Leptosynapta clarki during the reproductive cycle. A, C and E are photographs of fresh material. B, D and F are histological sections stained with haematoxylin and eosin at the same magnification (320x).

A. Mature ovary with thin tubule wall (Stage 1). Oocytes are contained within thin, clear tubule wall. Bar= 500 µm.

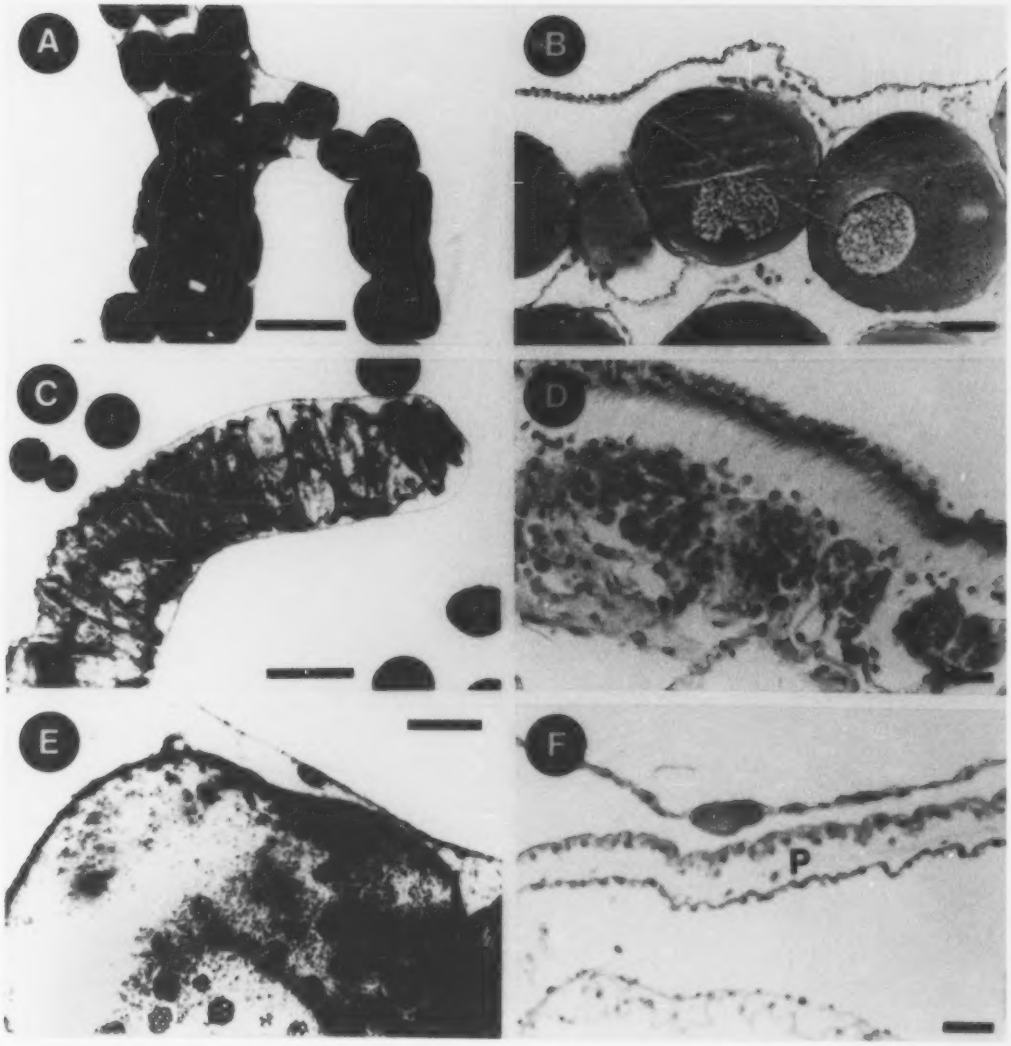
B. Histological section of Stage 1 ovarian wall. Oocytes are attached by follicular epithelium. Tubule wall is thin and flattened in section. Bar= 25 µm.

C. Prefertilization ovary with textured and thicker tubule wall (Stage 2). Tubule wall is opaque. Early stages of embryo development are free on the slide. Bar= 500 µm.

D. Histological section of Stage 2 ovarian wall. Tubule wall is thickened with extensions towards lumen. Note that droplets from degenerating oocytes are concentrated along ovarian wall. Bar= 25 µm.

E. Ovary in latter part of brooding period (Stage 3). Note posterior of pentactulae with spicules, thin tubule wall and developing oocyte. Bar= 200 µm.

F. Histological section of Stage 3 ovarian wall. As in E the posterior of a pentactula (p) is present. Ovarian wall is flattened with developing oocyte. Bar= 25 µm.


A
B
C
D
E
F
P

The second change in ovarian wall structure (Stage 3) occurred in the last months of brooding (Chapter 2). From February to May, some oocytes began to form in the germ layer of the tubule wall in the same region as large pentactulae (Fig. 3-1E). Histologically the ovarian wall returned to the thin flattened form seen in the period of oogenesis (Fig. 3-1F). Early oocytes were present adjacent to advanced pentactulae (Fig. 3-1F).

2. EM examination of ovarian wall.

Changes in the ovarian wall structure were examined using TEM in each of the three stages described above. The terminology utilized in the following descriptions follows that of Smiley and Cloney (1985) and Eckelbarger and Young (1992) in describing ovarian wall structure in holothurians. Three tissue layers are found in the ovarian wall: an outer visceral peritoneum, an inner germinal epithelium and a connective tissue compartment between the outer and inner layers. Each tissue layer will be considered separately, and the changes in structure within each stage noted in Section 3. Differences noted between the ovary and testis wall structure of Leptosynapta clarki (Atwood 1973) are noted in the descriptions of each layer.

a. Visceral peritoneum.

The peritoneum is the outermost layer of the ovarian wall and is in contact with the perivisceral coelom. The visceral peritoneum varies from squamous to low cuboidal epithelium and is composed of peritoneal cells, myoepithelial cells and neurons embedded in a loose basiepithelial nerve plexus (Fig. 3-2A, 3-2C, 3-2D, 3-3F).

Peritoneal cells possess a flattened nucleus, a few large and small electron dense granules, a Golgi body, mitochondria and occasional cisternae of rough endoplasmic reticulum (Fig. 3-2A, 3-2B, 3-2C, 3-2D, 3-3F). Numerous electron-dense granules were observed in some regions of peritoneal cells (Fig. 3-2B) as previously noted by Eckelbarger and Young (1992) and Atwood (1973) who described these as "secretory granules". The apicolateral margins of adjacent peritoneal cells are joined by septate junctions and the apex of each cell is adorned with microvilli and a single cilium (Fig. 3-2B, 3-2D, 3-3F). Peritoneal cells were also observed to contain microfilaments running parallel to the long axis of the cell.

Myoepithelial cells are confined to the peritoneum and lie over the basement membrane of the visceral peritoneum (Fig. 3-2A, 3-2C, 3-3C, 3-3E). Both circular and longitudinal fibres are present. Myoepithelial cells possess an oval to elliptical nucleus, numerous mitochondria and some electron-dense granules (Fig. 3-2C). The contractile

Figure 3-2. Transmission electron micrographs of Leptosynapta clarki ovaries: visceral peritoneum and connective tissue compartment.

A. Ovarian wall showing peritoneal cell (P), basal laminae (BL), connective tissue compartment (CT) and parietal cell (PC) adjacent to the ovarian lumen. Note the processes from myoepithelial cells (arrows). Bar= 2 µm.

B. Supporting cell of the peritoneum bearing a cilium (C). Cell contains vesicles and electron-dense granules. Bar= 1 µm.

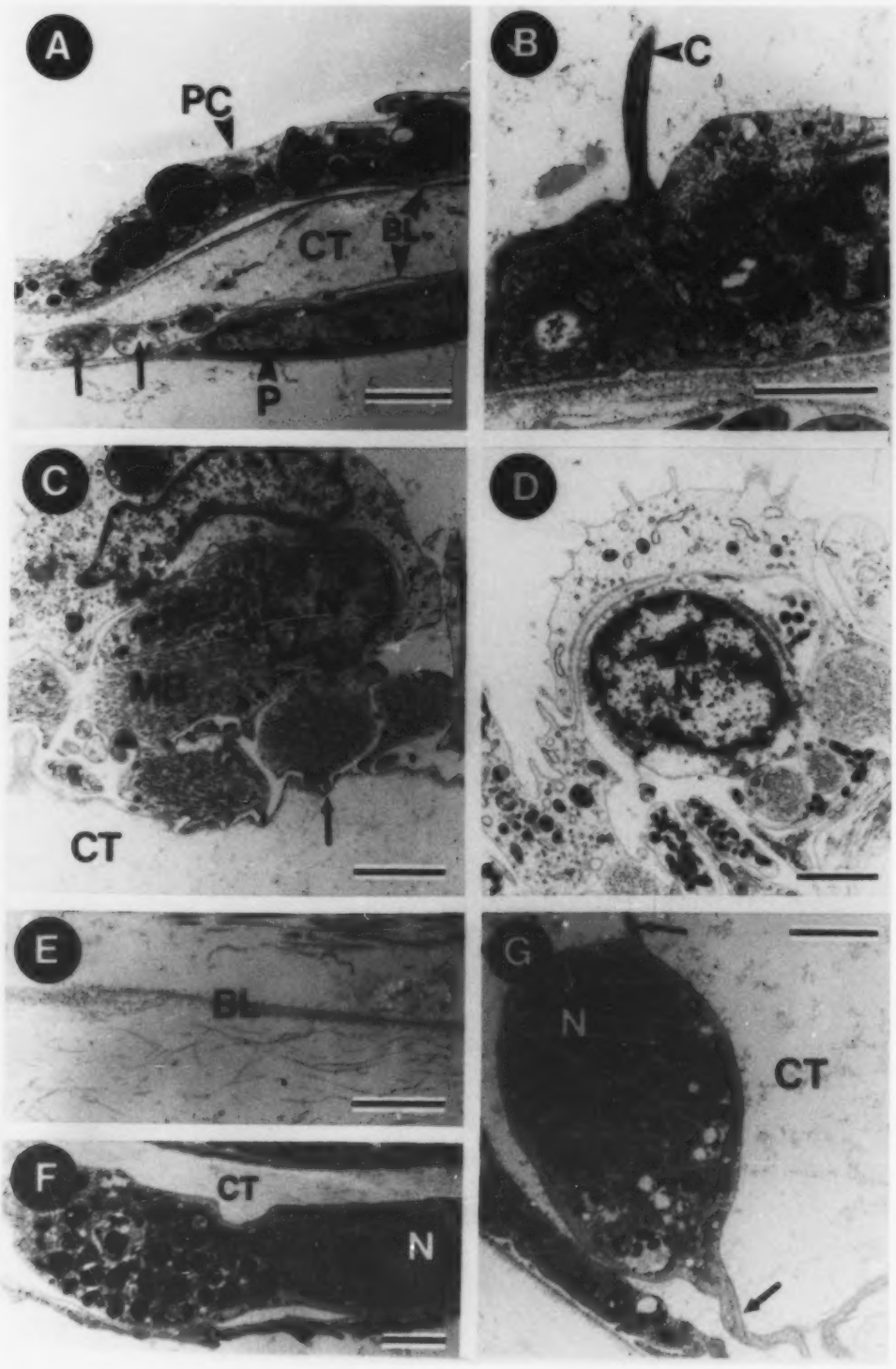
C. Myoepithelial cell with nucleus (N) and myofilament bundle (MB). Note the attachment of a myoepithelial cell to the basal lamina (arrow) of the connective tissue compartment (CT). Bar= 2 µm.

D. Neuron in peritoneum showing nucleus (N). Vesicles and rough endoplasmic reticulum are present in cell body. Bar= 2 µm.

E. Connective tissue compartment showing connective tissue fibres and basal lamina (BL). Bar= 2 µm.

F. Coelomocyte showing nucleus (N) with nucleolus and electron-dense granules in cytoplasm. Fibres of connective tissue compartment (CT) are also shown. Bar= 2 µm.

G. Fibroblast showing nucleus (N) with nucleolus. Cytoplasm contains electron-dense granules and vacuoles. Note the thin cytoplasmic processes from the cell (arrow) extending into connective tissue compartment (CT). Bar= 2 µm.


A
PC
CT
BL
P
B
C
C
ME
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BL
F
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N
G
N
CT

Figure 3-3. Transmission electron micrographs of Leptosynapta clarki ovaries: inner epithelium and changes in ovarian wall during reproduction.

A. Germinal epithelium showing parietal cell (PC) and developing oocyte with nucleus (N) and numerous mitochondria. Bar= 2.5 µm.

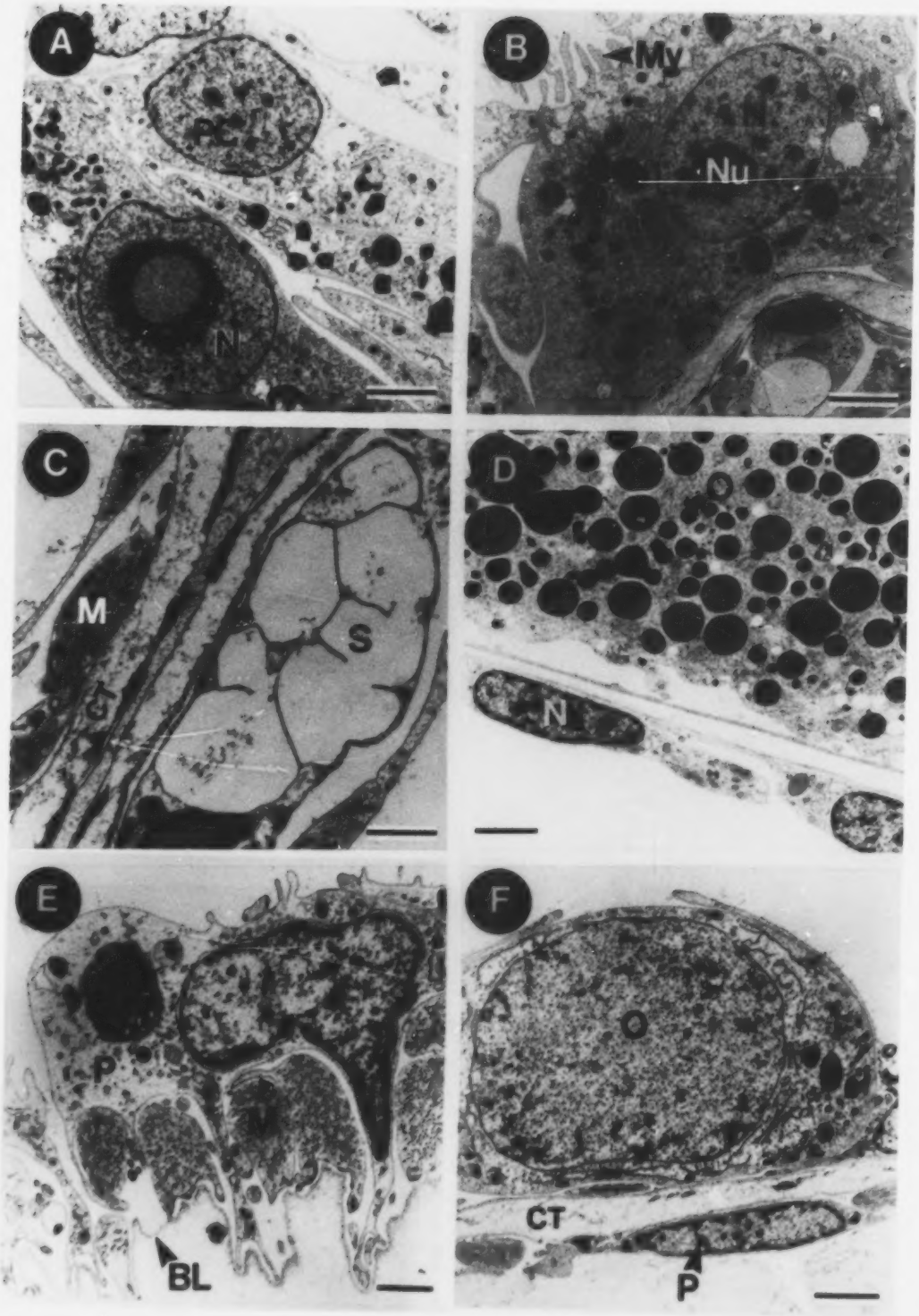
B. Parietal cell with nucleus (N), nucleolus (Nu) and microvilli at the apical surface (Mv). Cell contains mitochondria, Golgi bodies and electron-dense granules. Bar= 2.5 µm.

C. Ovarian wall germinal layer showing secretory cell (S) with vacuoles. Connective tissue compartment (CT) and myoepithelial cells of peritoneum (M) can also be seen. Bar= 1 µm.

D. Follicular cell with prominent nucleus (N) surrounding oocyte (O). Bar= 2.5 µm.

E. Arch-shaped peritoneal cell (P) with cytoplasmic extension that contacts the basal lamina (BL). Note the thick processes from myoepithelial cells (M) adjacent to the convoluted basal laminae. Bar= 1 µm.

F. Section through ovarian wall showing thin peritoneum (P), connective tissue compartment (CT) and germinal epithelium with developing oocyte (O). Bar= 2.5 µm.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apparatus is located to one side of the nucleus and is composed of myofilaments, each of which appear to be of uniform diameter measuring ca. 40 nm. Processes of myoepithelial cells attach to each other and to the subjacent basal lamina by hemidesmosomes (Fig. 3-2C, 3-3E).

Neurons and neurites contributing to the ovarian nerve are located towards the base of the peritoneum (Fig. 3-2C). Both are characterised by a variety of organelles including one or a combination of the following: clear vesicles measuring ca. 0.008 µm in diameter, homogeneous electron-dense vesicles measuring ca. 0.1 µm in diameter, microtubules or mitochondria. Neurites range from approximately 0.1-0.5 µm in diameter. Neuronal cell bodies possess a lightly staining cytoplasm, a few cisternae of rough endoplasmic reticulum, mitochondria and a spherical, centrally positioned nucleus (Fig. 3-2D).

Although electron-dense granules are seen in the peritoneal cells (Fig. 3-2B, 3-3E, Eckelbarger and Young 1992) no separate pigment cells as described by Atwood (1973) were observed in the visceral peritoneum.

b. Connective tissue compartment.

The connective tissue compartment is partitioned by the basal laminae of the peritoneum and the epithelium containing the germ line cells (Fig. 3-2A, 3-2E, 3-2F). This compartment creates the haemal sinus that houses a flocculent haemal fluid interspersed with connective tissue fibres (Fig. 3-2E, 3-2F, 3-2G). The connective tissue fibres are more numerous towards the basal lamina of the peritoneum (Fig. 3-2E, 3-2F) and appear to be collagen because they display periodic banding.

Mesenchymal cells, including fibroblasts and coelomocytes, are found within the connective tissue compartment. Coelomocytes are fusiform to circular in profile and contain numerous electron-dense granules measuring ca. 0.5 µm in diameter (Fig. 3-2F). The elliptical nucleus contains a nucleolus that is located either centrally or at one end of the cell. Coelomocytes also contain mitochondria, a Golgi body, electron-dense granules, and rough endoplasmic reticulum (Fig. 3-2F). Fibroblasts stain less densely and bear long cytoplasmic extensions (Fig. 3-2G). They possess a spherical nucleus with nucleolus, small electron-dense granules, vesicles and rough endoplasmic reticulum.

c. Inner epithelium.

The innermost layer of the ovarian tubule is composed of parietal cells, follicular cells, secretory cells and germ cells. Parietal cells and follicular cells form a continuous layer and are similar in contents. Differentiation between parietal and follicular cells is

based on the position of the cell with respect to the stalk of the oocyte: parietal cells line the lumen of the ovarian tubule up to the base of the oocyte stalk, while follicular cells surround each oocyte. Parietal cells are squamous to low cuboidal in shape and generally contain a spherical nucleus with a nucleolus (Fig. 3-3A, 3-3B). These cells are ciliated and contain large electron-dense granules up to 2 µm in diameter, inclusions resembling lysosomes, mitochondria and a Golgi body (Fig. 3-3A, 3-3B). Follicular cells are squamous in profile. They contain fewer electron-dense granules and more extensive rough endoplasmic reticulum as compared to parietal cells (Fig. 3-3D).

Secretory cells with vacuolated contents are also present in the parietal layer (Fig. 3-3C). The inclusions which characterise this cell type appear optically empty or contain fine flocculent material. They measure up to 5 µm in their greatest dimension. The secretory products dominate the contents of the cell and disperse the cytoplasm to the periphery regions of the cell (Fig. 3-3C).

The earliest germ cells observed were previtellogenic primary oocytes (Fig. 3-3A). These cells contain a large spherical nucleus with a doughnut-shaped nucleolus, numerous mitochondria, and several strands of rough endoplasmic reticulum. As oogenesis proceeds, the oocyte protrudes into the connective tissue compartment and becomes isolated by the stalk mentioned above. Further information on the process of oogenesis in holothurians is described in Smiley et al. (1991) and Eckelbarger and Young (1992).

3. Variations in the ovarian wall.

Changes in ovarian wall structure in different stages of reproductive activity are caused primarily by changes in the peritoneum (compare Fig. 3-2A and 3-3E). The cells of the peritoneum change in shape from thin flattened, squamous to convoluted cuboidal and arch shaped cells (Fig. 3-2C, 3-2D, 3-3E). The myoepithelial muscle cells become more pronounced and thickened; both longitudinal and circular muscles are affected (Fig. 3-3E). Junctional complexes are more numerous, especially those involving the muscle and peritoneal cells. Nerve cell processes (neurites) also become more obvious and concentrated, along with nerve cell bodies (Fig. 3-2D). The connective tissue layer also thickens so that there is an overall increase in the thickness of the ovarian wall from approximately 5 to 55 µm.

The thickening of the peritoneal epithelium and underlying connective tissue appears to be the result of the contraction of muscle cells as shown by their thickened processes (Fig. 3-3E). The nucleus and cytoplasm of the peritoneal cells extends through

the gaps between myoepithelial cells to reach the convoluted basal laminae of the connective tissue compartment (Fig. 3-3E).

During the latter part of the brooding period oogenesis may occur along the tubule wall. In regions where previtellogenic oocytes are forming the peritoneal cells are flattened and the connective tissue compartment is reduced in size (Fig. 3-3F). However, in other parts of the same cross-section the ovarian wall might still have the thickened texture seen in Figure 3-3E.

D. Discussion.

The functional significance of changes in ovarian wall structure during prefertilization and brooding in Leptosynapta clarki is at present unknown. The most profound change in ovarian wall structure during this period is in the size, density and configuration of the myoepithelial cells of the peritoneum and the overall increased thickness of the ovarian wall.

The myoepithelial cells of the ovary wall in holothurians are suggested to have two functions: routine gentle peristalsis of the tubules, and the strong contractions involved in spawning (Smiley et al. 1991). As female Leptosynapta clarki do not spawn the oocytes from the ovary there may an additional function for the myoepithelial cells to explain their extensive development prior to fertilization. Firstly, although the parietal cells lining the germinal epithelium are ciliated, the cilia are probably too small to move the viscous ovarian fluid. The extensions of the myoepithelial cells may, therefore, play a role in moving fluid within the ovarian tubules. This may be important in moving sperm through the ovary during fertilization, or in moving embryos within the tubule prior to hatching. The hatched pentactulae can move freely in the ovarian tubule (Chapter 2) and may avoid boundary layer constraints for oxygen diffusion by active movement themselves.

Alternatively the myoepithelial cells may act to increase the surface area of the ovarian wall. Unfertilized eggs in the Leptosynapta clarki ovary break into many discrete droplets which become free in the tubule after breakdown of the egg membrane (Chapter 2). These droplets are used for nutrition of developing pentactulae (Everingham 1961, McEuen 1986, Chapter 2) and tend to concentrate along the tubule wall adjacent to the regions of texture (Chapter 2, Fig. 3-1D). It is possible that these droplets are incorporated into the ovarian haemal system, and then used by the mother for her own nutrition or for gametogenic growth (Chapter 2).

Peritoneal cells of holothurian ovaries have been suggested to take up nutrients from the coelomic fluid (Smiley and Cloney 1985, Smiley 1988). Arch-shaped peritoneal cells with processes that penetrate the underlying myoepithelial cells in the ovaries of two deep-sea holothurians may permit unidirectional nutrient flow from the peritoneum towards the ovarian lumen during vitellogenesis (Eckelbarger and Young 1992). Basal projections of the peritoneal cells around the myoepithelial cells seen in Leptosynapta clarki (Eckelbarger and Young 1992) are also observed in this study (Fig. 3-3E). In the present study, however, the extensive projections of the peritoneal cells were observed in the mature or brooding ovary after vitellogenesis was complete. This suggests that while nutrient flow might be towards the ovarian lumen during vitellogenesis, it is possible that there is movement of nutrient, waste or oxygen in both directions during brooding.

In the latter part of the brooding period but before pentactulae release (April/May, Everingham 1961, McEuen 1986, Chapter 2) the ovarian wall returned to its thin flattened form. The reduction in ovarian wall thickness associated with this change may facilitate the release of single pentactulae or the breaking of the distal part of the ovarian tubule into the coelom (Chapter 2) and subsequent escape from the mother by autotomy or rupture of the body wall (Chapter 6). The decrease in size of the myoepithelial cells also flattens the germinal epithelium and new oocyte growth begins in the same part of the tubule as the pentactulae (Chapter 2).

Atwood (1973) noted that the basic structure of the gonadal wall in Leptosynapta clarki was identical in the testis and ovary. However, some differences were noted between the ovary in our study and Atwood's (1973) description of the Leptosynapta clarki testis. For example, we observed no pigment cells in the peritoneum and all peritoneal cells appeared to be ciliated, although Atwood (1973) noted that cilia rarely projected into the coelom.

Differences between gonadal wall structure in Leptosynapta clarki with sex was unexpected as this species is a protandric hermaphrodite (Chapter 2, Chapter 4). Males in the process of sex change have oocytes growing in the germinal epithelium while mature or remnant spermatozoa fill the tubule lumen (Chapter 2, Chapter 4). Therefore, the germinal epithelium of the same gonad tubule produces both spermatogonia or oogonia at different phases of an animal's lifetime. The minor differences in structure noted between the ovary and testis (Atwood 1973) may result from differences in the location of study (Bamfield vs False Bay, San Juan Island, Washington), the fact that Atwood's samples were taken at a single time of year or the difference in function between the ovary and testis.

Until further ultrastructural studies are made of holothurian ovaries during the reproductive season, it is unknown whether the changes in ovarian wall structure observed here are related to the additional function of the ovary as a brood structure or also occur in broadcast spawning species. The finding that there can be significant morphological and structural changes in the ovarian wall during the reproductive period does, however, caution the use of single samples in ultrastructural studies.

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CHAPTER 4

Small size, brooding and protandry in the apodid sea cucumber Leptosynapta clarki.

A. Introduction.

Two characters of the adult have been associated with brooding in marine invertebrates: small size and the tendency towards hermaphroditism. Several hypotheses have been proposed to explain the association of small adult size with brooding. The energetic hypothesis of Chia (1974) suggested that the trend for lecithotrophy, brooding, or viviparity in small animals resulted from the lower energetic reserves for gamete production. Strathmann and Strathmann (1982) described three categories of hypotheses on the association of small adult size and brooding related to: 1) dispersal, 2) adult longevity and recruitment, and 3) the allometry of structures associated with gamete production and brood care. These hypotheses are not mutually exclusive and neither hypothesis is entirely satisfactory (Strathmann and Strathmann 1982). However, of these four hypotheses the allometry hypothesis has received the most attention, primarily as it can be tested within a single species.

The allometry hypothesis predicts that brooding does not occur in large species because of spatial limitations on brood size with increasing animal size (Heath 1977, Strathmann and Strathmann 1982). In numerical terms, while fecundity is likely to increase with the cube of length, the space for brooding may increase with a lower scaling constant resulting in insufficient space for brooding the embryos. Tests of this hypothesis involve a comparison of the relationship between animal size, egg number and number of brooded embryos, and have been made in several marine invertebrates (Daly 1972, Rutherford 1973, Menge 1974, Ockelmann and Muus 1978, Rumrill 1982, Strathmann et al. 1984, Kabat 1985, Gremare and Olive 1986, McGrath and ÓFoighil 1986, Russell and Huelsenbeck 1989, Byrne 1991, Brey and Hain 1992, Hines 1992, Hess 1993).

Hermaphroditism, where both male and female gametes are produced by a single individual in its lifetime, occurs commonly in marine invertebrates and is often associated with brooding (Ghiselin 1969, 1974). Ghiselin (1969, 1974) proposed three models for the evolution of hermaphroditism: 1) The low density model proposes that in sessile or slow moving species, or in low density populations, simultaneous hermaphroditism is advantageous as each contact of mature individuals can lead to

reproduction (Tomlinson 1966, Ghiselin 1969). 2) The size-advantage model proposes that sequential hermaphroditism will occur where an individual reproduces most efficiently as a member of one sex when small or young, but as a member of the other sex when it gets older and larger. Originally proposed by Ghiselin (1969) this has been extensively modelled (e.g. Warner 1975, Leigh et al. 1976, Charnov 1982, 1986, Iwasa 1991). 3) The gene dispersal model proposes that where motility is low, both the number and variety of potential mates are limited, and the genetic environment may be affected. Ghiselin (1969) discusses two particular effects: sequential hermaphroditism could reduce sibling crosses (Ghiselin 1969), while simultaneous hermaphroditism may increase effective population size (Murray 1964).

More recently, Heath (1977, 1979) has predicted that if the space for brooding was limited and could hold fewer embryos than the individual could produce, then the remainder of reproductive resources could be reallocated towards male function as a simultaneous hermaphrodite. Data collected in Asterina phylactica were inconsistent with this hypothesis and led Strathmann et al. (1984) to propose that the correlation between brooding and simultaneous hermaphroditism was related to levels of inbreeding.

While most brooding echinoderms are simultaneous hermaphrodites, recent research has suggested an association between brooding and sequential hermaphroditism within this phylum. In a comprehensive review of sex changing species, Policansky (1982) cited evidence that 10 species of echinoderms change sex, the majority being protandric; two of these are brooders (Table 4-1). Since this review a protandric brooding ophiuroid (Byrne 1991) and a protandric non-brooding asteroid (Byrne 1992) have been described (Table 4-1). There are also one brooding and two non-brooding echinoderms that may change sex (Table 4-1).

Leptosynapta clarki is a small (maximum length 113 mm), apodid sea cucumber found on the intertidal mudflats of Grappler Inlet, Bamfield, Vancouver Island, Canada. L. clarki has an annual reproductive cycle, brooding pentactulae in the ovary from November to April or May (Everingham 1961, Chapter 2). Abundant sperm found in the ovaries of Leptosynapta clarki sampled in False Bay, San Juan Island, Washington, in March or April (Everingham 1961, Eckelbarger and Young 1992) led Everingham to suggest that this species might be a protandric hermaphrodite. The present study confirms that Leptosynapta clarki is protandric, describes the population consequences of that sex change, and tests the allometry hypothesis for association of small adult size and brooding in marine invertebrates.

Table 4-1. Patterns of sex change and reproductive characters in the Phylum Echinodermata. Information compiled from cited references; presence or absence of brooding based on review of Giese et al. (1991).

Species	Sex change	Brood	Evidence for sex change	
Class Asteroidea				
Fromia ghardaqana	Protandric	N	H	Achituv and Delavault 1972
Asterias forbesi	Protandric	N	L	Aldrich and Aldrich 1955
Asterina gibbosa	Protandric	N	F^2, H	Bacci 1951
	Protogynic	N	?	Bacci 1965
	Protandric	N	H	Emson and Crump 1978
Asterina pancerii	Protogynic	?	H	Cognetti 1954
Patiriella exigua	Protandric	N	F^1, F^2	Byrne 1992
Class Holothuroidea				
Holothuria atra	Protandric	N	F^1, F^2	Harriott 1982
Lapidoplax media	Protandric	?	F^1	Gotto and Gotto 1972
Leptosynapta inhaerens	Protandric	N	F^1	Runnström 1927
Leptosynapta clarki	Protandric	Y	H; F^1, F^2, H	Everingham 1961; herein
Class Ophiuroidea				
Ophiolepis kieri	Protandric	Y	F^1, F^2	Hendler 1979
Amphiura stepanovii	Protandric	Y		Fedotov 1926
Ophiocantha bidentata	Protandric	N	F^2, H	Tyler and Gage 1982
Ophionereis olivacea	Protandric	Y	F^2, H.	Byrne 1991

Brood: Y= yes; N= no; ?= unknown. F= Field collections, F^1 = skewed sex ratio, F^2 = size/sex relationship; H= Histology; L= Laboratory observations. **Suspected species**: Brooder: Pseudopsolus macquariensis Simpson 1982. Non-brooder: Ophioplocus (Ophiceres) incipiens Mortensen 1936, Ophiothrix fragilis Gostan 1956.

B. Materials and methods.

1. Collections and measurements of size.

Leptosynapta clarki were collected at two sites in Grappler Inlet, Bamfield, British Columbia (Site 1: 48°49'57" N, 125°06'45" W; Site 2: 48°50' N, 125°06'36" W). Site 1 is located on the northern side of Barge Bay, has a short intertidal area (ca. 20 m) and a high proportion of gravel in the substrate. Site 2 is a gentler sloping mudflat (intertidal area ca. 60 m) at the opposite end of the channel separating "No-name" Island from Vancouver Island proper (see Chapter 2, Fig. 2-1). This site has a finer sediment composition with little gravel and contains Leptosynapta clarki of a larger size than Site 1 (Mean total weight ± SD: Site1: 307.8 ± 153.99 mg, N=720; Site 2: 490.4 ± 268.80 mg, N=660, Chapter 2). Both sites have extensive Zostera sp. beds in the lower intertidal that extend into the shallow subtidal.

Samples of Leptosynapta clarki were collected on the mid-intertidal mudflats using cores or by sweeping away the upper 5 cm of sediment to reveal the animals in their burrows. The sea cucumbers were collected with the surrounding mud and transported to the laboratory in buckets. Adult Leptosynapta clarki were removed from the sediment using a 2 mm or 0.85 mm sieve. The remaining fraction was then sieved through a 0.25 mm sieve to remove juveniles. Sea cucumbers were kept in sea water until measurement (length and weight) after relaxation in 2.5% $MgCl_2$ in sea water (w/v).

Measurements of length (mm) were made from the anterior end of the calcareous ring to the posterior while in $MgCl_2$ in a 90 mm petri dish. Sea cucumbers less than 20 mm were measured on a dissecting microscope using an eyepiece graticule calibrated against a stage micrometer. Larger sea cucumbers were gently held straight with tweezers and length measured to the nearest millimetre with reference to a small plastic ruler under the petri dish.

After measurement of length, the sea cucumber was placed on a tissue to remove external water, and then weighed on a fine scale balance (= total weight TW, in mg).

2. Size-sex relationship.

The relationship between size and sex in Leptosynapta clarki was determined in July 1991 when the gonads were well developed (Chapter 2). After weighing, the sex of each sea cucumber was determined by examining the colour of the gonads through the semi-transparent body wall. In July, females had yellow ovaries with large eggs

(ca. 200 µm) and males white testes (Chapter 2). Dissections were made to verify identification in juvenile sea cucumbers or where gonads were small.

The relationship between sea cucumber size and sex was determined by calculating the percent of females in each weight or length class. For weights less than 800 mg the data was divided into 50 mg size classes. Low numbers of sea cucumbers were found above 800 mg, so the size class was increased to 100 mg. The final category was sea cucumbers with weights > 1300 mg. Sea cucumbers in this category ranged from 1412 to 1684 mg (N=4), so are plotted as a single point at 1500 mg. Sea cucumber length was divided into 5 mm size classes until 100 mm, then the three sea cucumbers with greater length (104, 108, 113 mm) are plotted as a single point at 110 mm. The total number of sea cucumbers sampled was 693 (454 males, 239 females).

The breeding sex ratio was determined from haphazard cores used in following the recruitment of Leptosynapta clarki on the intertidal mudflats at Site 1 (Chapter 6). Ten haphazardly thrown cores were taken on the mid-intertidal in Barge Bay (Chapter 6), the animals removed by sieving, and the size and sex determined of each sea cucumber in four samples during the winter of 1990-1991 and the summer of 1991.

3. Gonadal changes.

Sea cucumbers sampled during regular gonad index dissections in January and February of 1990 and 1991 (Chapter 2) were used to determine the size of animals with transitional gonads (= in the process of changing from male to female). Sea cucumbers were collected from Sites 1 and 2, weighed and dissected as described in Chapter 2 (Total N=240). The gonad in Leptosynapta clarki consists of two separate tubules that join through the dorsal mesentery to a single gonoduct (Chapter 2). Both gonad tubules were dissected, placed on a glass slide (38 x 75 mm) with a small amount of sea water and temporarily mounted with a coverslip (35 x 50 x 0.02 mm). The weight of the coverslip revealed details of the gonad, such as the presence of oocytes, without the need for histology. Of the 240 sea cucumbers dissected 87 were females brooding pentactulae or undergoing resorption. Of the remaining 153 sea cucumbers, seventy five had transitional gonads; these were divided into 50 mg size classes for analysis.

Histological descriptions of protandric sex change were obtained during a detailed study of reproduction in Leptosynapta clarki (Chapter 2). Gonads were preserved in Bouin's fixative, stored in 70% ethanol and embedded in Paraplast. Sections were cut at 7 µm, stained in haematoxylin and eosin, and photographed on a Zeiss photomicroscope.

4. Allometry of reproduction.

The relationship between the size of the female and the number of unfertilized eggs was determined in late October 1989 and 1990 prior to the spawning period in November and December (Everingham 1961, McEuen 1986, Chapter 2). Sea cucumbers were collected from Sites 1 and 2 and sieved from the sediment as described above. After measuring total weight (TW), a longitudinal incision was made from the base of the calcareous ring to the posterior, the coelomic water drained from the body cavity and the animal reweighed (= drained weight in mg, DW). The gonad was dissected and temporarily mounted under a coverslip. Counts were made of the total number of mature eggs per gonad (N= 2 tubules) in 20 females at two sites in each year (Total N= 80 females).

The relationship between the size of the female and the number of fertilized eggs/embryos was examined in sea cucumbers in late November and early December 1989 and mid-November 1990. This was during the spawning period of Leptosynapta clarki at Bamfield (Chapter 2) but before degeneration of unfertilized eggs or significant pentactulae mortality (Chapter 5). Counts were made of the number of unfertilized eggs, early stage embryos, or pentactulae in each gonad for 50 females. The allometric relationship between female size and number of pentactulae was determined for those females that had early embryos or pentactulae present (N= 28).

The allometric relationship between size and number of eggs or pentactulae was tested using a Model II Reduced Major Axis (RMA) regression. Ordinary Least Squares (OLS) regressions were calculated using Microsoft Excel Version 3.0. Because of the considerable error in measuring size in sea cucumbers (Sewell 1990), OLS regression will under-estimate the value of the slope (see McArdle 1988, LaBarbera 1989), and may suggest scaling constraints exist in cases when there are none (Hess 1993). RMA regressions were performed on raw and natural log ($\log_e$) transformed data. Standard error (s^2_β) and assymetric confidence intervals were attached to the slope of the RMA regression using the formulas in McArdle (1988).

If there are no constraints on brood size the number of embryos brooded is assumed to scale with body size in the same way as the fecundity of broadcast spawning animals; i.e., linearly with body weight or the cube of length (Strathmann and Strathmann 1982). To test whether brood size was limited by scaling constraints, the slope of the RMA line was thus compared to the slope predicted by isometry (b=1 for the relationship between $\log_e$ body weight and $\log_e$ number of pentactulae) using a t-test (Clarke 1980, McArdle 1988). A slope in this relationship less than 1 would indicate scaling constraints on brood size in larger sea cucumbers.

C. Results.

1. Size-sex relationship.

Leptosynapta clarki is a protandric hermaphrodite with sex change occurring over a broad range of size (Fig. 4-1). Sea cucumbers in the smallest weight class (0-50 mg) are juveniles of the year (Chapter 6) and are all male (Fig. 4-1A). The smallest females found weighed 77, 120, and 150 mg (21, 36, 30 mm in length respectively, Fig. 4-1).

Sex change did not occur in a distinct weight class (Fig. 4-1A). Few females were found until 200-250 mg total weight (Fig. 4-1A). Between 200 and 400 mg there was an increasing percentage of females in each size class. This percentage stabilised at approximately 50% at weights above 500 mg (Fig. 4-1A).

A similar increase in percent females was seen with increased sea cucumber length (Fig. 4-1B). No females were found until the 20-25 mm size class. This was followed by an increasing percentage of females between 25 and 45 mm that plateaued at 50% above a length of about 50 mm (Fig. 4-1B).

The presence of large numbers of small-sized, but sexually active, males in the population strongly biases the sex ratio in Leptosynapta clarki (Table 4-2). In all samples the sex ratio was above 70% male with a maximum of 88.5% male in August (Table 4-2).

2. Gonadal changes.

The majority of sea cucumbers with transitional gonads in January or February were in the 200-400 mg size range (Fig. 4-2). Sex change is, however, observed in some very large sea cucumbers (Fig. 4-2). The largest sea cucumber found with a transitional gonad weighed 855 mg total weight.

After spawning the testes became thin and yellow in colour, and shrank in length towards the gonad basis (Chapter 2). In protandric males new oocytes were observed in fresh gonad tubules at the basal end adjacent to the gonad basis (Fig. 4-3A). Unspawned sperm were often present in the tubules with the new oocytes (Fig. 4-3B) and were active when exposed to sea water.

Histological examination of male gonads revealed that oocytes were present along the tubule wall in some mature testes (Fig. 4-3C). The testis lumen was packed with mature spermatozoa, while previtellogenic oocytes 10 to 20 µm were found along the tubule wall (Fig. 4-3C). After spawning of the spermatozoa, the oocytes continued to grow while the remnant spermatozoa were resorbed (Fig. 4-3D). The "decision" to

Figure 4-1. Percent female Leptosynapta clarki from Grappler Inlet, Bamfield in size classes of A. Total weight (mg) and B. Length (mm). Point is plotted at upper limit of size class except for final category shown with (x); see details in text.

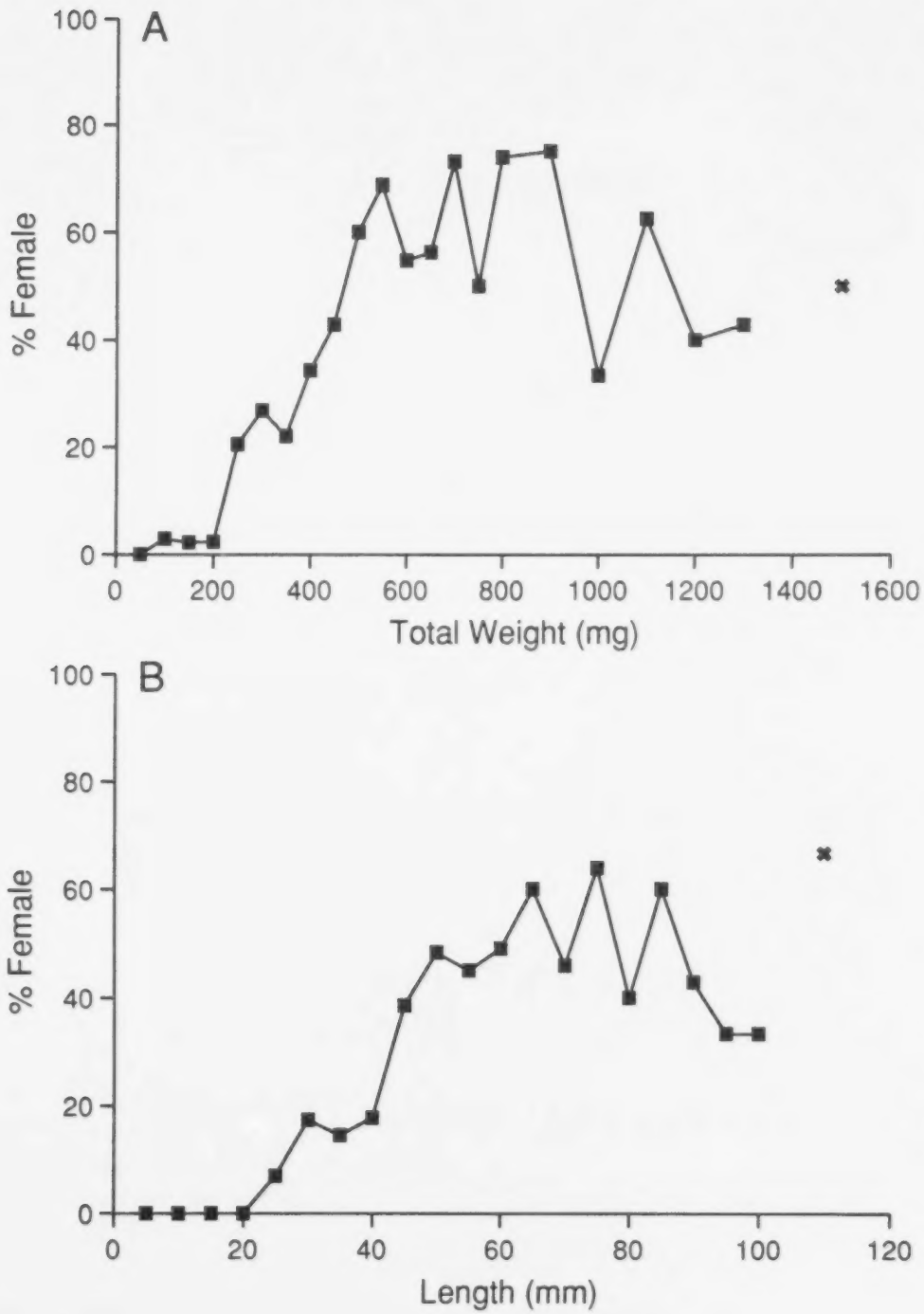

A
100
80
60
40
20
0
% Female
0
200
400
600
800
1000
1200
1400
1600
Total Weight (mg)
B
100
80
60
40
20
0
% Female
0
20
40
60
80
100
120
Length (mm)

Table 4-2. Breeding sex ratio of Leptosynapta clarki at Site 1, Grappler Inlet.

DATE	% MALE	SEX RATIO Male:Female	N
7 December 1990	70.5	2.4:1	112
7 February 1991	85.3	5.8:1	143
15 July 1991	85.9	6.1:1	128
12 August 1991	88.5	7.7:1	96
Mean	82.6		
Std. Deviation	8.2		

Figure 4-2. Number of transitional Leptosynapta clarki gonads found in each 50 mg total weight class during January and February 1990 and 1991. N= 75 sea cucumbers.

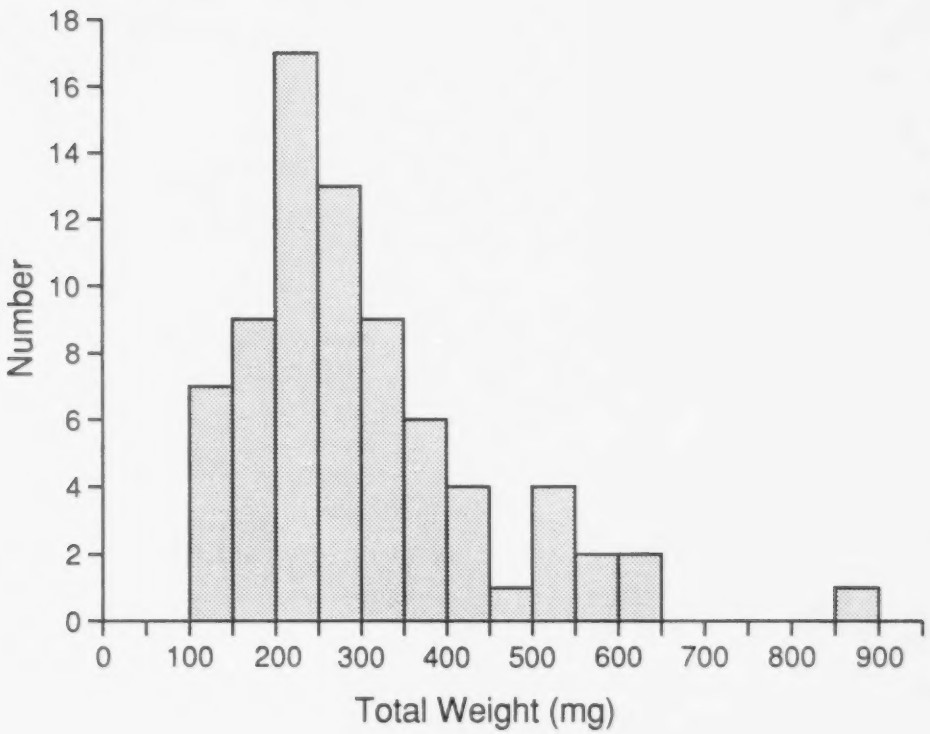

18
16
14
12
10
8
6
4
2
0
Number
0
100
200
300
400
500
600
700
800
900
Total Weight (mg)

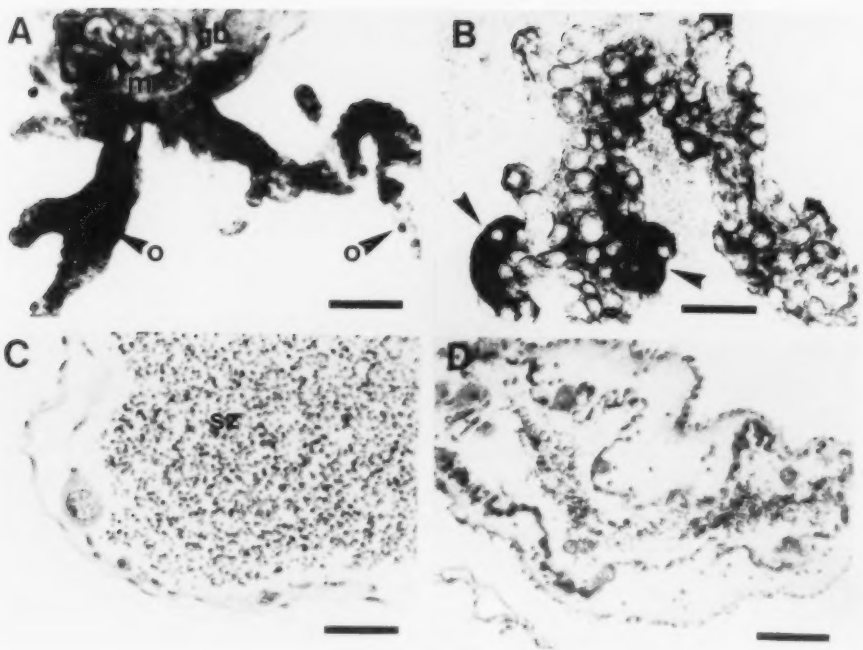
Figure 4-3. Transitional gonads in Leptosynapta clarki.

A. Photograph of small resorbing testis in process of sex change. Almost the entire gonad is shown. Note the swollen gonad basis (gb) with madreporite (m), and small oocytes (o) in the tubules. Bar= 500 µm.

B. Photograph of transitional testis. Tubule contains numerous oocytes and remnant packets of sperm (arrow). Some sperm has broken from tubule onto base of slide. Bar= 200 µm.

C. Histological section of mature testis with spermatozoa (sz) in the lumen and early oocytes forming along the tubule wall. Section stained with haematoxylin/eosin. Bar= 25 µm.

D. Histological section of post-spawned testis in transitional stage. Some remnant spermatozoa are seen in the lumen. Oocytes are present along the tubule wall. Bar= 50 µm.


A
bb
m
o
o
B
C
se
D

change sex must, therefore, be made prior to spawning in the current reproductive season.

3. Allometry of reproduction.

Reduced major axis (RMA) regressions were calculated between female size, number of eggs or pentactulae using raw and $\log_e$ transformed variables (Table 4-3). In the period prior to spawning, female sea cucumbers show a positive significant relationship between weight (TW or DW) and egg number (Fig. 4-4, Table 4-3). Regressions using natural log transformations of these variables are also significant, although there is a slight reduction of the r^2 in both cases (Table 4-3). The allometric exponent of the $\log_e$-transformed egg number/drained weight relationship is 1.46, with 95% confidence intervals being 1.3-1.65 (Table 4-3).

Lengths of the females used in the egg number/weight relationship were not recorded. These lengths were estimated using the relationship between total weight and length determined from the size/sex relationship described above using OLS regression (Length= 0.0595(TW) + 20.519, F= 2393.37, df = 1, 691, $p< 0.0001$, $r^2 = 0.776$). For this analysis OLS regression is appropriate as the value for length is being estimated from the measurement of another variable (LaBarbera 1989). A significant relationship was found between length and number of eggs in raw and natural log regressions, with an allometric exponent of 2.24 in the $\log_e$-transformation (Table 4-3).

Weak relationships were found between female size and the number of pentactulae brooded (Table 4-3). The only significant relationship was between drained weight and number of pentactulae in raw and $\log_e$-transformed data (Table 4-3). Both regressions have low r^2 values (< 0.4, Table 4-3). The allometric exponent of the $\log_e$-transformed regression was 1.84 (Table 4-3). This slope is significantly greater than 1 (T= 4.772, d.f.= 26, $p< 0.001$) indicating that fecundity is not proportionately smaller in larger sea cucumbers. In fact, larger females are producing disproportionately larger broods. This suggests that there are no scaling constraints on brood size in Leptosynapta clarki.

Fertilization success in female Leptosynapta clarki was variable. During the period sampled 22 of the 50 females (44%) were unfertilized. The remainder had percent fertilization ranging from 4.18-98.49% (mean ± SD = 47.74 ± 25.37%) with the mode at 40-50% fertilization (Fig. 4-5). There was no relationship between female weight and the percent fertilization (TW: F= 0.193, df = 1, 26, NS; DW: F= 0.986, df = 1, 26, NS).

Table 4-3. Scaling constants for relationships between Leptosynapta clarki size and number of eggs or pentactulae. Relationships are determined from an RMA regression on raw or $\log_e$ transformed data. Formula for standard error ($s^2\beta$) and confidence intervals (β lower, β upper) on the RMA slope from McArdle (1988). TW= total weight (mg), DW= drained weight (mg).

Regression	N	F	r^2	ln(a)	b	$s^2\beta$	β lower	β upper
TW vs # eggs	80	168.94^{***}	0.684	-12.300	3.637	0.038	3.206	4.127
DW vs # eggs	80	377.55^{***}	0.829	-237.726	6.348	0.071	5.785	6.968
Length vs # eggs	80	168.94^{***}	0.684	-1050.37	61.169	0.775	53.911	69.405
ln TW vs ln # eggs	80	161.69^{***}	0.675	-0.010	1.416	9.5×10^{-3}	1.246	1.610
ln DW vs ln # eggs	80	205.89^{***}	0.725	0.114	1.463	9.5×10^{-3}	1.300	1.646
ln Length vs ln # eggs	80	138.53^{***}	0.640	-0.589	2.465	0.011	2.154	2.821
TW vs # pentactulae	28	0.143 NS	0.005					
DW vs # pentactulae	28	15.590^{***}	0.375	44.960	1.795	0.055	1.312	2.456
Length vs # pentactulae	28	0.143 NS	0.005					
ln TW vs ln # pentactulae	28	0.184 NS	0.007					
ln DW vs ln # pentactulae	28	6.644^{**}	0.204	1.069	1.835	0.063	1.289	2.611
ln Length vs ln # pentactulae	28	0.122 NS	0.005					

*** $p < 0.001$; ** $p < 0.05$

Figure 4-4. Relationship between drained weight (mg) and number of eggs (filled squares) or pentactulae (open squares) in Leptosynapta clarki.
RMA regressions: Eggs: $y = 6.348x - 237.726$, $r^2 = 0.829$, N=80.
Pentactulae: $y = 1.795x + 44.960$, $r^2 = 0.375$, N= 28.

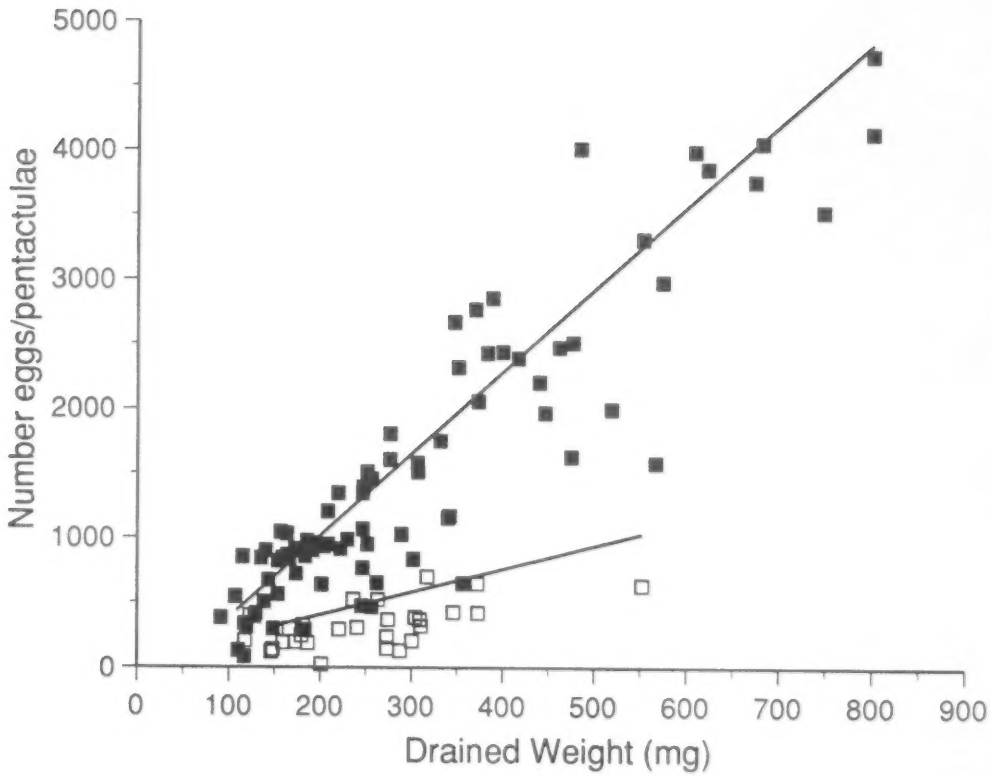
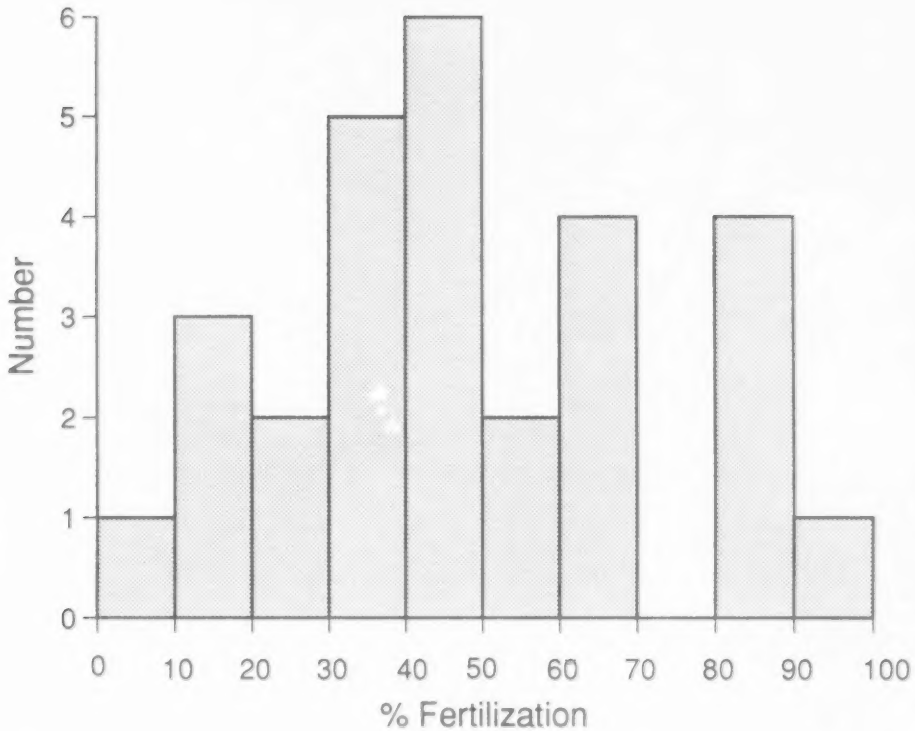

5000
4000
3000
2000
1000
0
Number eggs/pentactulae
0
100
200
300
400
500
600
700
800
900
Drained Weight (mg)

Figure 4-5. Number of female Leptosynapta clarki in each percent fertilization class during November and December 1989 and 1990. N= 28 females.


Number
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
80
90
100
% Fertilization

D. Discussion.

1. Protandry in Leptosynapta clarki.

The histological and population information presented here confirms that Leptosynapta clarki is a protandric hermaphrodite and provides the first direct evidence of sex change within the Class Holothuroidea. Two other holothurians in the Order Apodida have been suggested to be protandric, though the evidence in both cases is somewhat circumstantial. Leptosynapta inhaerens was believed to be protandric as in 80 spawning specimens only 4 shed eggs (Runnström 1927, cited in Hyman 1955), while the lack of males in a sampled population of Labidoplax media led Gotto and Gotto (1972) to suggest that only very small and young specimens produce sperm.

There are few reports of sex change in the five remaining holothurian orders. There are some anecdotal reports of protandry among dendrochirote holothurians (Smiley et al. 1991), but protandry has not been clearly demonstrated in any species. In aspidochirote holothurians, protandry is suggested in Holothuria atra based on a change in the sex ratio with increased size (Harriott 1982). However, sex change in H. atra is complicated because of asexual reproduction by binary fission (Harriott 1982). In addition, evidence for sex change in H. atra may be difficult to obtain as gonad regression after each spawning makes determination of the previous sex impossible (Harriott 1982).

Sex allocation theory predicts that sex change should be an "all or nothing" response, with the animal or plant producing gametes of one sex until it is more profitable to produce gametes of the other sex, and then switching entirely to the latter (Charnov 1982). Research on marine invertebrates has, however, shown a number of species that do not show an "all or nothing" response but have a 1:1 sex ratio above a certain size (Patella argenvillei Branch 1981, Lottia gigantea Wright 1989, Patella kermadecensis Creese et al. 1990, present study), or a wide size range or time over which sex change occurs (Fromia ghardaqana Achituv and Delavault 1972, Ophionereis olivacea Byrne 1991, Coralliophila violacea Soong and Chen 1991, Patiriella exigua Byrne 1992). The debate continues as to whether this is the result of a genetically programmed sex change, environmental sex determination, or both.

One explanation applied in a number of marine invertebrates is that there are two sexual races within a species. Bacci (1951), who proposed this terminology in Asterina gibbosa, described one race whose individuals change sex at a specific time in their existence (= balanced hermaphrodites). In the other race, sex change occurs earlier or later depending on the individual (= unbalanced hermaphrodites), or an individual may

remain male or female for its entire lifespan (= true males or females, Bacci 1951). In the latter race, the presence of "true" males or females suggests a genetic predetermination for that sex. Evidence for this is seen in the echiuran Bonellia viridis where 10% of the larvae develop as males even if the "male determining factors" of a female proboscis are absent (Leutert 1975). Similarly, some individuals of Patella vulgata may never change sex (Orton 1928, Ballantine 1961) and in Lottia gigantea about 15% of the population change sex annually regardless of environmental setting or age (Lindberg and Wright 1985).

In Leptosynapta clarki, as all individuals start their reproductive life as males (Sewell 1991, Chapter 2, Chapter 6), there can only be "true" males in the population that do not change sex during their lifetime. Division of the remainder of the population must be into those who change sex at some specific time, and those that change sex under the influence of some genetic or environmental factor. Male Leptosynapta clarki may either delay sex change as shown by the large size of some transitional males (Fig. 4-2) or have a labile sexuality which alternates between male and female. There is some circumstantial evidence that sex can be reversed from female to male (Chapter 2), and it is conceivable that sex may change more than once in an holothurian's lifetime (Everingham 1961, Harriott 1982).

The presence of transitional Leptosynapta clarki gonads containing mature sperm and previtellogenic eggs during the reproductive season (Fig. 4-3) and the continued growth of oocytes within the same gonad tubule after spawning of the sperm (Chapter 2) makes it difficult to envisage how a genetic factor is involved in the control of sex change. The ability to maintain gametes from two sexes, selectively resorb the remnant sperm, and continue to grow oocytes suggests some form of complex control. As little is known of sex determination in echinoderms (Delavault 1966) and especially unknown in holothurians (Smiley et al. 1991) this could be a fruitful area for further research.

The size-sex relationship presented here is a static data set which combines sea cucumbers of many ages within a size class (Fig. 4-1). Juveniles released from the mother in May 1990 were maintained in the laboratory for 1 year and used for measurements of growth (Chapter 6). These individuals reproduced as males in November 1990 (Chapter 6). When the growth experiment finished in May 1991 the surviving sea cucumbers were kept in the laboratory until the gonads were grown. In July 1991, eight animals of the surviving 78 had changed sex (unpub. data). These protandric sea cucumbers were above 185 mg TW (unpub. data) which is approximately the critical size of 200-250 mg determined from field collections. The other 70 individuals, while of the same age, were male or of undetermined sex (unpub. data). Seven of these 70 sea

cucumbers were above 185 mg TW but had not changed sex (unpub. data). These results suggest, therefore, that size in combination with some environmental or genetic factor may be more important than age per se to the timing of sex change in Leptosynapta clarki.

2. Brooding and small body size.

Brooding is size-dependent in many marine invertebrates, with species with smaller adults providing protection to more advanced stages of embryonic development (see review by Strathmann 1990). This trend has been noted in two families of holothurians, the Cucumaridae (Menge 1975) and the Synaptidae (Strathmann 1990). Leptosynapta clarki conforms to these trends in being small (adult size max. at Bamfield 113 mm), and brooding pentactulae in the ovary (Everingham 1961, McEuen 1986, Chapter 2).

A test of the allometry hypothesis to explain the association of small adult size with brooding (Strathmann and Strathmann 1982) in Leptosynapta clarki did not show proportionately smaller broods in larger sea cucumbers. The slope of the relationship between $\log_e$ drained weight and $\log_e$ number of pentactulae was not less than one, indicating no spatial constraints on brood size in larger Leptosynapta clarki.

Constraints on brood size can also occur if the larger broods from larger adults develop more slowly or suffer higher mortality (Strathmann and Strathmann 1982). In Leptosynapta clarki while there is a trend for larger females to have a higher number of pentactulae, larger females or females with a high number of pentactulae do not show a higher degree of mortality (Chapter 5). These results suggest that brood size is not constrained in larger Leptosynapta clarki by either brood space or differential embryonic mortality.

While the allometry hypothesis is intuitively appealing (Hess 1993), the evidence for scaling constraints on brood size in marine invertebrates is remarkably poor. Studies that suggest that brood size is unconstrained by brood space include species of polychaetes (Daly 1972, Gremare and Olive 1986, Hess 1993), bivalves (Ockelmann and Muus 1978, Kabat 1985, McGrath and ÓFoighil 1986, Russell and Huelsenbeck 1989, Brey and Hain 1992), asteroids (Menge 1974), ophiuroids (Rumrill 1982, Byrne 1991), holothuroids (Rutherford 1973) and pinnothurid crabs (Hines 1992). In fact, the study on Asterina phylactica which provided the first empirical evidence for the hypothesis (Strathmann et al. 1984) when reanalysed with RMA regression does not indicate any scaling constraints on brooding (Hess 1993).

One species that might show allometric constraints on brooding is the hermaphroditic bursal brooding Axiognathus squamata (Rumrill 1982). In this species the relationship between brood number and parental body size is significantly different from zero, but with a low slope (untransformed data b=0.57, Rumrill 1982). This indicates that smaller sized adults brood nearly equivalent numbers of embryos as larger adults (Rumrill 1982). However, in Axiognathus squamata staggered oocyte maturation and brood release ensures that the production of oocytes never exceeds the capacity of the adult to brood the developing embryos and thus clutch size depends more upon the rate of oocyte maturation than body size per se (Rumrill 1982).

Fertilization in brooding marine invertebrates is by three methods: self-fertilization, some form of intromittant device is employed, or sperm are shed externally into sea water and are attracted towards the eggs retained by the other parent (Heath 1977). The latter method will encourage the maximum production of male gametes since large numbers of ova may be available for fertilization (Heath 1977). Poor relationships between the number of embryos brooded and parental size seen in internal (e.g. Axiognathus squamata Rumrill 1982, Leptosynapta clarki present study) or external brooders (Engstrom 1982) could result from problems associated with fertilization in all females regardless of size.

Increased fertilization success in large sea cucumbers might be observed if there is an advantage in large size in competition for breeding sites or mates, or intense sperm competition (Conover 1984). Little is known about spawning behaviour in Leptosynapta clarki (Chapter 2). Although Everingham (1961) and McEuen (1986) reported seeing individuals lying close together or intertwined on the mud surface during the spawning period, this behaviour was not observed at Bamfield (Chapter 2). As neither of these authors observed spawning or found spermatozoa in the depressions formed by intertwined sea cucumbers, it is unknown whether these groups are in fact formed by spawning individuals (Chapter 2). Until the spawning behaviour of Leptosynapta clarki is determined it is assumed that there is no competition for breeding sites or mates in this species. Sperm competition, however, might be intense.

In Leptosynapta clarki the males release sperm externally which enters the ovary through an as yet unknown means (Everingham 1961, McEuen 1987, Hess et al. 1988, Chapter 2), but probably via the gonopore. Sperm availability during the spawning season is maximised in two ways: by early reproduction which skews the sex ratio dramatically towards males (Table 4-2), and the production of large quantities of sperm by each male (Chapter 2). While the males invest a similar proportion of energy in reproduction (ca. 5% in all sized males, Chapter 2), larger males will have larger

volumes of sperm which might result in higher fertilization success. However, even though sperm does not appear to be limiting because of high density populations (mean 169 per m^2, Chapter 6), a skewed sex ratio, and large amounts of sperm per male, in some females fertilization is low or zero. There is also no evidence to suggest that larger females have a higher fertilization success. If fertilization is a constraint for Leptosynapta clarki, or other internal brooders, then limitations on brood size due to space or embryonic requirements for dissolved materials (Strathmann and Strathmann 1982) may never be reached.

In brooding species where fertilization success is high (e.g. Rutherford 1973, Ophioplocus esmarki Rumrill 1982, Byrne 1991) allometric constraints may be avoided by brooding embryos to a limited stage of development (Daly 1972, Ockelmann and Muus 1978), three-dimensional packing of embryos in the brood space (Kabat 1985), sequential brooding throughout the reproductive season (Kabat 1985, Hess 1993), or brooding within a distensible structure (Hines 1992, present study). In the latter case, the ability for the brood structure to expand to accommodate the number of fertilized eggs might ensure that even when fertilization success is high all the embryos can be brooded.

The alternative hypotheses on the association of small size and brooding such as lower energetic reserves (Chia 1974), dispersal or recruitment (Strathmann and Strathmann 1982) cannot be considered in relation to brooding in Leptosynapta clarki until there is comparative information on other sea cucumber species. Hess (1993) has recently suggested that the slower developmental rate of embryos in large brooders may be a factor in the evolution of brooding. This time constraint hypothesis suggests that longer developmental times may be disadvantageous because the stage specific mortality rate is experienced over a longer period of time, and the number of broods per unit time is reduced. Hess (1993) assumes in the development of this hypothesis that embryonic mortality may be low and relatively unimportant in brooders, which may not be a valid assumption in all brooding species (Chapter 5).

While the time constraint hypothesis may be applicable to species that spawn more than once per season, alternative hypotheses will be needed to explain the association of small size and brooding in semelparous brooders or individuals that produce only one brood per season (Hess 1993). As the latter category includes many echinoderms in which the association of small size and brooding is particularly pronounced (see Strathmann 1990), this emphasises that no single hypothesis will be applicable in all taxa (Strathmann and Strathmann 1982).

3. Brooding and hermaphroditism.

The association between brooding and hermaphroditism has been noted in a variety of marine invertebrates (Ghiselin 1969, 1974, Charnov 1982, Strathmann and Strathmann 1982), including asteroid, ophiuroid and holothuroid echinoderms (Ghiselin 1969, 1974). The fairly high incidence of protandry in ophiuroids and holothuroids was believed by Ghiselin (1969) to favour the gene dispersal model for hermaphroditism.

In the gene dispersal model, sequential hermaphroditism can act to prevent inbreeding due to fertilization between siblings, especially in forms with restricted gene flow (Ghiselin 1969, 1974). For example, if all members of a clutch are males at the onset of sexual maturity, and simultaneously change into females then they are never able to mate with each other (Ghiselin 1974). The observation that there is a higher incidence of sequential hermaphroditism in forms with restricted gene flow (species with no larvae or brooders) provides some evidence for the hypothesis (Ghiselin 1974) though no direct evidence for reduced levels of inbreeding in sequential hermaphrodites is available. Studies on the scale of genetic differentiation in Leptosynapta clarki (Hess et al. 1988) and the confirmation of protandry in the present study may provide direct evidence for this hypothesis.

In an electrophoretic study of three geographically separate Leptosynapta clarki populations on San Juan Island, Washington, Hess et al. (1988) found no evidence for inbreeding. The two polymorphic loci studied did not show the reduced numbers of heterozygotes expected in inbred populations (Hess et al. 1988). Hess et al. (1988) concluded that there was enough movement of either individuals or gametes within each population to allow random mating and maintain heterozygosity.

Leptosynapta clarki juveniles released from the mother rapidly form shallow burrows and although dispersal is rare it might occur by rafting, floating, swimming or incidental dispersal as a result of water movement (Chapter 6). The combination of occasional dispersal and sequential hermaphroditism might reduce levels of inbreeding in a Leptosynapta clarki population. However, as growth rates in juvenile Leptosynapta clarki are extremely variable (Chapter 6), and protandry appears to be dependent upon the combined effects of size and environmental/genetic effects it is unlikely that all siblings undergo a simultaneous sex change. While Leptosynapta clarki does show some evidence for the gene-dispersal model for sequential hermaphroditism further clarification is required to determine how far siblings disperse from one another, if all or only some individuals in a clutch change sex at one point in time, and if there is a reduction in inbreeding in the Bamfield population.

If we assume that sequential hermaphroditism is an advantageous strategy for Leptosynapta clarki, the question to be answered is: why is Leptosynapta clarki protandric and not protogynic?

Juvenile sea cucumbers are released from the mother into the adult habitat in April or May (Chapter 2, Chapter 6). The transitional gonads with new eggs are, however, seen in sex-changing animals in January while these pentactulae were still being brooded. Therefore, if the sea cucumbers are to be reproductively active as females in their first year, they would need to start egg development while being brooded within the female. In addition, based on the regression line for drained weight versus number of eggs (Table 4-3, Fig. 4-4) to produce any eggs at all a sea cucumber must weigh over 37 mg DW during late October. While growth to sizes at which eggs could be produced is possible based on laboratory measurements (Chapter 6), the time required to produce mature eggs (10 months, Jan-Nov., Chapter 2) or the energy to support oogenesis (Lawrence 1987) might be a constraint to early reproduction as a female. If energy is limited, there may be a size threshold below which it is uneconomical to produce the energetically more expensive eggs (Branch 1981) but where the energy is adequate to produce a functional number of smaller sperm (Lawrence 1987).

In terms of the size advantage hypothesis, while there is clearly an increase in the number of eggs produced per female with size (Fig. 4-4), the potential for low fertilization (as number of eggs fertilized) in all females may reduce any advantage in large females. In addition, there is a strong relationship between testis weight and male size (unpub. data) so larger males are producing larger volumes of sperm. Until the mechanism of fertilization in Leptosynapta clarki is determined, it is not known whether male size is important to an individual's fertilization success.

4. Future research.

The genus Leptosynapta provides an ideal group in which to conduct further research on the association of small size, hermaphroditism and brooding in marine invertebrates. Sea cucumbers within this genus are of small to moderate size (Hyman 1955; 12-30 cm maximum adult length, except for L. minuta 1 cm, McEuen 1986), are characterised by burrowing habits (Hyman 1955), and exhibit a wide range of reproductive strategies from lecithotrophic broadcast spawners (L. inhaerens, L. galliennei, L. girardii, L. tenuis), to coelomic (L. minuta), and ovarian brooders (=viviparity: L. clarki, L. transgressor; for references see Smiley et al. 1991). Of these species L. clarki and probably L. inhaerens are protandric, while L. minuta is reported to be hermaphroditic (Smiley et al. 1991). Further comparative work on this genus would

therefore be of considerable interest to address life history questions relating small size, brooding, and hermaphroditism without phylogenetic constraints.

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CHAPTER 5

Mortality of pentactulae during intraovarian brooding in the apodid sea cucumber Leptosynapta clarki.

A. Introduction.

In 1950, the Danish marine biologist Gunnar Thorson published a review that provided the basis for much of our theory in marine invertebrate larval ecology (Roughgarden 1989, Young 1990). Because of the low number of eggs produced by species with non-feeding larvae or brooders, Thorson suggested that "... embryos developing into non-pelagic young are free from the huge waste found among larvae with a pelagic stage" (Thorson 1950, p.10). He also believed that the best protection for embryos during development was given by species with viviparity or with highly organised brood protection (Thorson 1950).

The assumption that mortality during brooding is lower than in planktotrophic species has been generally accepted when discussing life history theory (e.g. Mileikovsky 1971, Vance 1973a, 1973b, Chia 1974, Strathmann 1985, Emlet et al. 1987). Chia (1974) further postulated that larval mortality increased with the number of eggs produced, and in non-feeding forms increased with the distance of larval development from the female (viviparity → brooded → benthic → pelagic).

Determination of natural mortality rates in marine invertebrate larvae has presented a formidable challenge to scientists for many years (Rumrill 1990). Although there are numerous estimates of larval mortality in planktonic species (see reviews: Chaffee and Strathmann 1984, Strathmann 1985, Hines 1986, Rumrill 1990) there are few studies that focus on mortality during encapsulation or brooding (see Rumrill 1990, Rawlings 1990). These and other authors have noted the need for estimates of mortality in non-pelagic species, and the importance of estimating "the relationship between the energy put into brood protection and the reduction in mortality which results" (Grant 1983, p. 554).

There is some evidence of mortality during brooding in asteroids (Menge 1974, Hendler and Franz 1982, Strathmann et al. 1984) and ophiuroids (Rumrill 1982), and prior to hatching in holothuroids (Rutherford 1973). However, these studies make assumptions about fertilization success which may be inappropriate and result in inaccurate estimates of mortality. Menge (1974) based his initial embryo number on the regression between egg number and female body size, assuming 100% fertilization. This

assumption may not be valid in broadcast spawning species (e.g., Pennington 1985, Levitan et al. 1992, Sewell and Levitan 1992 and references therein) and is generally unknown in brooders. Fertilization rates of less than 100% will result in under-estimates of survival and over-estimate instantaneous mortality (Rumrill 1990). The remaining studies on mortality assume that there is the same degree of fertilization in each female, and estimate mortality based on the difference in the number of early and late stage embryos in different females (Hendler and Franz 1982, Strathmann et al. 1984, Rumrill 1982) or consider only pre-hatching mortality (Rutherford 1973).

Accurate estimates of mortality during brooding can only be obtained if the initial number of embryos can be determined. A unique situation for measuring embryo mortality was found in the intraovarian brooding sea cucumber Leptosynapta clarki. This species broods pentactulae in the ovarian tubules (= viviparity, Komatsu et al. 1990, Wourms and Callard 1992) from November to April or May (Everingham 1961, Chapter 2). During the pentactula stage a calcareous ring is present at the base of the tentacles (Everingham 1961, Chapter 2). While death of the pentactula results in the resorption of the embryonic tissues, the calcareous ring remains visible in the ovarian tubule. Counts can thus be made of the numbers of living and dead pentactulae and the mortality rate determined for each female. This chapter reports mortality in Leptosynapta clarki pentactulae throughout the brooding period and suggests that mortality in a viviparous species can be extremely high.

B. Materials and Methods.

Adult Leptosynapta clarki were collected at two sites in Grappler Inlet, Bamfield, British Columbia (Site 1: 48°49'57" N, 125°06'45" W; Site 2: 48°50' N, 125°06'36" W). Site 1 is located on the northern side of Barge Bay, has a short intertidal area (ca. 20 m) and a high proportion of gravel in the substrate. Site 2 is a gentler sloping mudflat (intertidal area ca. 60 m) at the opposite end of the channel separating "No-name" Island from Vancouver Island proper (see Chapter 2, Fig. 2-1). This site has a finer sediment composition with little gravel and contains Leptosynapta clarki of a larger size than Site 1 (Mean total weight ± SD: Site1: 307.8 ± 153.99 mg, N=720; Site 2: 490.4 ± 268.80 mg, N=660, Chapter 2). Both sites have extensive Zostera sp. beds in the lower intertidal that extend into the shallow subtidal.

Leptosynapta clarki were collected from the mid-intertidal at each site, transported in buckets of mud to the laboratory and kept overnight in running sea water. Sea cucumbers were removed from the sediment using a 0.85 mm sieve and placed in a

bowl of sea water. Female sea cucumbers that had not autotomized their posterior ends were chosen at random from the bowl and relaxed in 2.5% $MgCl_2$ in sea water (w/v) for 10-15 minutes. After relaxation the sea cucumber was placed on a tissue to remove external water and weighed on a fine-scale balance (= total weight in mg). A longitudinal incision was then made from the base of the calcareous ring to the posterior, the coelomic water drained from the body cavity and the sea cucumber reweighed (= drained weight).

The ovary of Leptosynapta clarki is located in the anterior coelom and consists of two gonad tubules, one to each side of the dorsal mesentery (Chapter 2). The two gonad tubules were dissected from the coelom of each female under a binocular microscope, placed on a glass slide (38 x 75 mm) with a small amount of sea water and temporarily mounted with a coverslip (35 x 50 x 0.02 mm). The weight of the coverslip revealed the pentactulae within the ovary without damaging the tubule. The slide was placed under a dissecting microscope and the number of living pentactulae, dead pentactulae or calcareous rings were recorded for each female. A conservative attitude was adopted in counting a pentactula as dead; in tubules where there was extensive resorption of the dead pentactulae the number of calcareous rings were counted. Photographs of ovaries with dead pentactulae were taken on a Leitz compound microscope immediately after counting was completed.

Samples of female Leptosynapta clarki were taken at Sites 1 and 2 at monthly intervals throughout the brooding period (Nov.-May, Chapter 2). Ten females were dissected from each site at each time interval. However, not all females were brooding pentactulae so the number of females on which mortality is based varied between 1 and 10 at each site. In February 1991, mortality estimates are also included from females brooding pentactulae in gonad index dissections (Chapter 2).

Relationships between features of the mother (female size, number of pentactulae brooded) and percent mortality were tested by combining data from Sites 1 and 2 for each month. As additional pentactulae mortality might occur after sampling, the data for each month were not pooled for analysis. Ordinary Least Squares (OLS) regressions were calculated using Microsoft Excel Version 3.0.

C. Results.

1. Mortality estimates.

Embryonic development in Leptosynapta clarki has been described by Everingham (1961) at False Bay, San Juan Island, Washington. Hatching occurs after

the prepentactula stage to form a 5-tentacled pentactula which is able to move freely in the ovarian tubule (Everingham 1961, Chapter 2). Development to the pentactula stage occurs in approximately 1-2 weeks (Chapter 2), so the majority of the brooding period is spent as a pentactula (Chapter 2).

During dissections, dead pentactulae were observed in the ovarian tubules. Dead pentactulae had a discoloured body wall due to early resorption (Fig. 5-1A), closed tentacles and an incompletely connected gut (Fig. 5-1B), or the pentactula was in a contorted position. In more advanced stages of resorption the body of the pentactula was a "ghost" around the calcareous ring (Fig. 5-1C), or the calcareous ring was the only structure remaining in the ovarian tubule (Fig. 5-1D). By totalling the number of living pentactulae, dead pentactulae and calcareous rings the initial number of brooded pentactulae could be determined and mortality calculated.

Mortality of pentactulae during brooding varied between females, ranging from 0-100% (Table 5-1, Fig. 5-2). During the early part of the brooding period, the percent mortality was generally low but it increased over time (Table 5-1, Fig. 5-2). Differences in mortality were also seen between sites and years. Site 2 had a higher mean percent mortality in all samples in both years (Table 5-1), and the mean percent mortality was higher in each month during 1990-1991 than in 1989-1990 (Table 5-1). The number of females that had lost the entire brood was also higher in the 1990-1991 reproductive season (1989-1990: 1 female; 1990-1991: 8 females; Fig. 5-2). This reproductive season was characterised by its unusually cold conditions in December and January (Days with temperatures below 0°C in Dec.and Jan.: 1989-1990= 9 d; 1990-1991= 25 d, P. Janitas unpub. data) and on many days the intertidal was overlain with discontinuous ice sheets (pers. obs.).

Females that had 100% mortality ranged in size from 141-671 mg total weight (mean ± SD: 372 ± 180 mg, N= 9)and are within the size range of females with <100% mortality (82-1161 mg total weight, mean ± SD: 368 ± 184 mg, N=146). However, females with 100% mortality were brooding a lower number of pentactulae (mean ± SD: 30.7 ± 34.7 pentactulae, range 1-99, N= 9) than females with <100% mortality (mean ± SD: 173.5 ± 172.9 pentactulae, range 1- 839, N=146). Two females contained only a single dead pentactula.

2. Instantaneous mortality.

Estimates of instantaneous mortality (day^{-1}) were calculated for pentactulae brooded in female Leptosynapta clarki in March and April (1990, 1991) in the period

Figure 5-1. Photomicrographs of Leptosynapta clarki ovarian tubules during the brooding period.

A. Dead pentactula with discoloured body wall. Bar= 500 µm.

B. Anterior end of dead pentactula showing calcareous ring (cr) at base of tentacles and resorbing intestine (i). Bar= 500 µm.

C. Dead pentactula with extensive resorption of the soft body tissues. Calcareous ring is visible within "ghost" of body. Bar= 500 µm.

D. Complete resorption of dead pentactula. Calcareous ring is the only remaining structure. Bar= 200 µm.

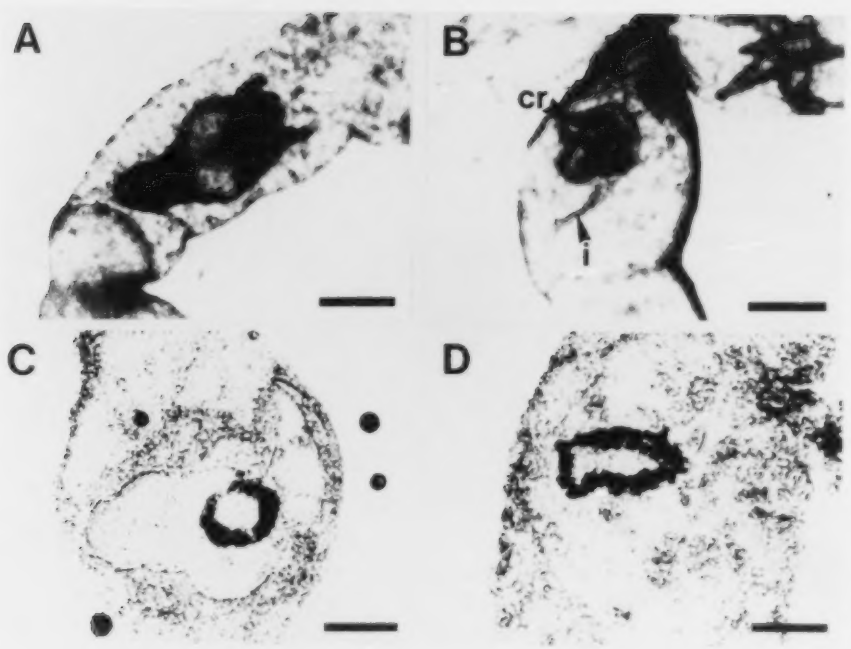

A
B
cr
i
C
D

Table 5-1. Mean percent mortality of brooded pentactulae in female Leptosynapta clarki. Samples were taken at Site 1 and Site 2 in the 1989/1990 and 1990/1991 seasons. Sample size (N) refers to the number of females in which pentactulae were present.

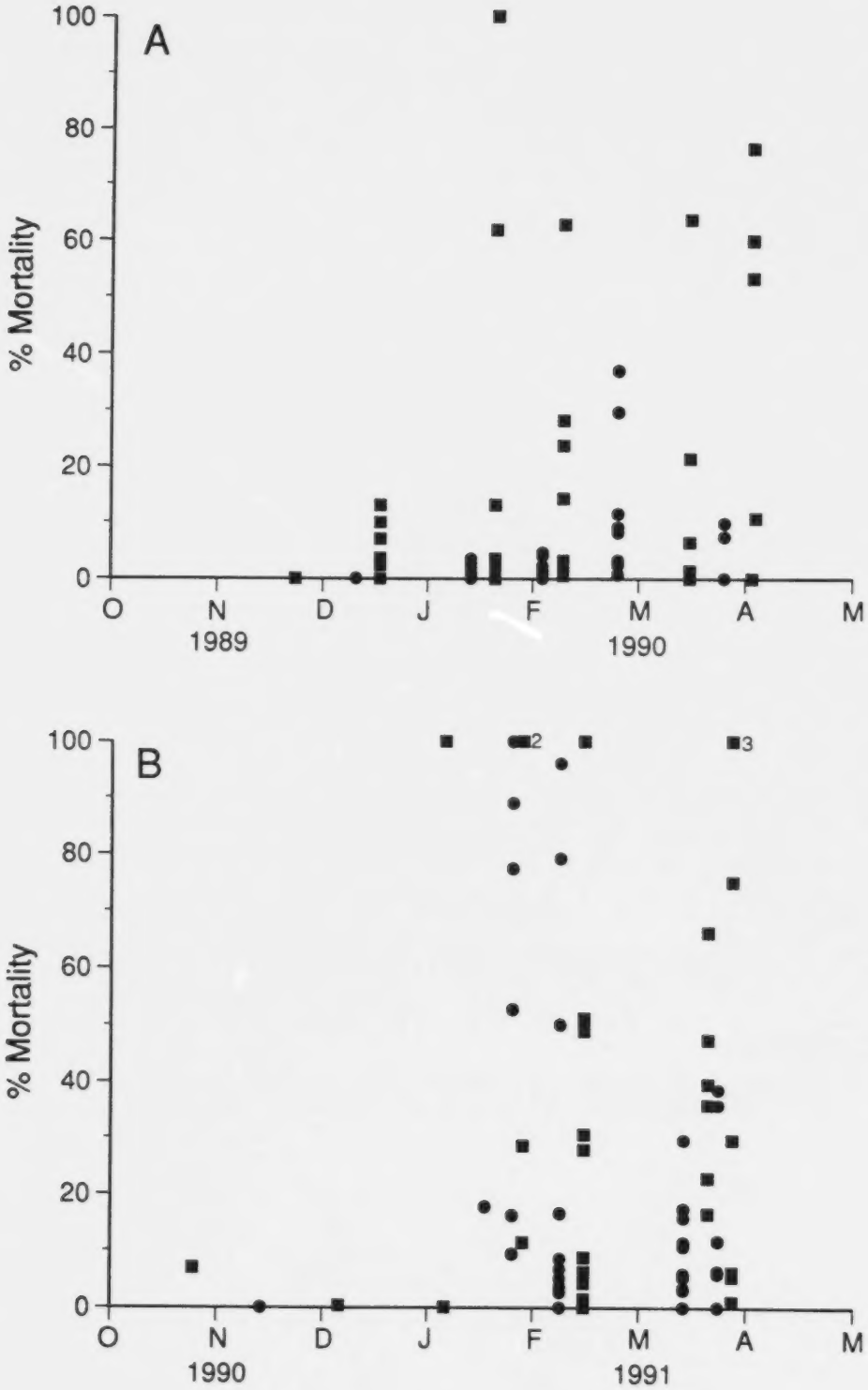
Site 1:

DATE	MEAN	SD	N	RANGE
16 December 1989	0	0	4	
19 January 1990	1.27	1.10	10	0 - 2.70
9 February 1990	1.59	1.76	9	0 - 4.62
3 March 1990	10.54	12.72	10	0.65 - 37.04
3 April 1990	5.77	5.12	3	0 - 9.8
18 November 1990	0	-	1	
24 January 1991	17.56	-	1	
1 February 1991	57.37	38.06	6	9.35 - 100
25 February 1991	22.93	33.23	12	0 - 96.08
23 March 1991	9.53	8.61	11	0 - 29.41
2 April 1991	12.24	15.88	8	0 - 38.46

Site 2:

DATE	MEAN	SD	N	RANGE
23 November 1989	0	0	5	
18 December 1989	4.31	4.72	9	0 - 13.04
21 January 1990	25.85	39.46	7	0 - 100
10 February 1990	15.14	20.83	9	0.51 - 62.86
4 March 1990	11.61	22.28	8	0 - 63.64
6 April 1990	40.10	33.04	5	0 - 76.47
24 October 1990	6.9	-	1	
6 December 1990	0.31	-	1	
17 January 1991	50.00	70.71	2	0 - 100
30 January 1991	59.92	46.80	4	11.32 - 100
27 February 1991	20.66	28.93	14	0 - 100
25 March 1991	37.95	17.83	6	16.42 - 66.15
1 April 1991	46.98	45.74	9	0.93 - 100

Figure 5-2. Percent mortality of pentactulae during brooding in Leptosynapta clarki at Site 1 (circles) and Site 2 (squares). A. Mortality in the 1989-1990 reproductive season. B. Mortality in the 1990-1991 reproductive season. Points for Site 1 are offset by five days to avoid overlapping points. In B, numbers adjacent to values of 100% mortality describe the number of observations with that value.


A
% Mortality
100
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1989
1990
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F
M
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M
1990
1991

prior to pentactulae release (Everingham 1961, Chapter 2). The formula used was as defined in Strathmann (1985) and Rumrill (1990):

$$M = \ln (N_0 / N_t) / -t$$

assuming that fertilization occurred on December 1st of each year. This date was chosen as it is approximately the centre of the spawning period in Leptosynapta clarki at Bamfield (Chapter 2).

Values for instantaneous mortality in Leptosynapta clarki are low (Table 5-2) primarily because of the long brooding period (t = 93-128 days). Ten females had zero mortality (M=0). Three females had lost their entire brood (i.e. $N_0 / N_t = 0$) and the value of M could not be calculated. For comparison to other species the grand mean for females with some living pentactulae is -0.0021 day^{-1} (SD= 0.0030, N=57).

3. Mortality in relation to features of the mother.

No clear relationships were seen between the percent mortality and features of the mother such as size (drained weight) or number of pentactulae brooded (Table 5-3). The relationship between percent mortality and the size of the female was non-significant for each sampling date except March 1991 (Table 5-3). At this time the largest females had the highest percent mortality (Fig. 5-3).

The relationship between percent mortality and the total number of pentactulae brooded was similarly significant in January 1990 and February 1991 only (Table 5-3, Fig. 5-4). In both cases the r^2 for the OLS regression is less than 0.3 (Table 5-3) and there is considerable scatter in the relationship (Fig. 5-4). In these months there is a trend for the highest percent mortality in those females brooding the fewest pentactulae (Fig. 5-4).

D. Discussion.

The retention of the calcareous ring of dead pentactulae in the ovarian tubules Leptosynapta clarki has provided a unique system for studying mortality in a viviparous marine invertebrate as the number of pentactulae beginning the brooding period can be determined. As the calcareous rings or pentactulae are retained within a discrete structure, there is no error due to the loss of embryos during sampling (as in Leptasterias tenera, Hendler and Franz 1982). Additionally, as the pentactulae are released from the ovary at the same point in time (Chapter 2) errors associated with the release (Rumrill 1982) or migration of early maturing juveniles (Hendler and Franz 1982) are avoided.

Table 5-2. Instantaneous mortality rates (M, day^{-1}) for pentactulae brooded in female Leptosynapta clarki in March and April 1990, 1991. Formula as defined in text and assuming the beginning of the brooding period as December 1st.

Date	mean M	SD	minimum M	maximum M	N
March 1990	-0.0015	0.0027	-0.0108	0	18
April 1990	-0.0034	0.0043	-0.0114	0	8
March 1991	-0.0022	0.0025	-0.0094	0	17
April 1991	-0.0018	0.0031	-0.0114	0	14 *

* Three females had no living embryos (i.e. 100% mortality). Values for M could not be calculated as $(N_0 / N_t) = 0$ and are omitted from the mean value for M.

Table 5-3. Ordinary Least Squares regressions between female size (drained weight), total pentactulae number and observed mortality (%). Data points from Sites 1 and 2 are combined for each sampling period in 1990 and 1991. Values given are F-statistic (F) and r^2 for regression. Significance of regression: NS = nonsignificant, * $p < 0.05$, ** $p < 0.01$.

	Drained Weight (mg)		Total Pentactulae Number		
Date	F	r^2	F	r^2	N
January 1990	0.016 NS	0.001	4.851 *	0.244	17
February 1990	0.094 NS	0.006	2.889 NS	0.152	18
March 1990	0.0004 NS	2.3×10^{-5}	0.297 NS	0.018	18
April 1990	2.140 NS	0.263	0.761 NS	0.113	8
January 1991	2.659 NS	0.347	0.165 NS	0.032	7
February 1991	1.231 NS	0.039	9.268 **	0.236	32
March 1991	5.325 *	0.262	2.117 NS	0.124	17
April 1991	0.030 NS	0.002	2.164 NS	0.126	17

Figure 5-3. Relationship between drained weight and percent mortality of pentactulae brooded in female Leptosynapta clarki in March 1991.

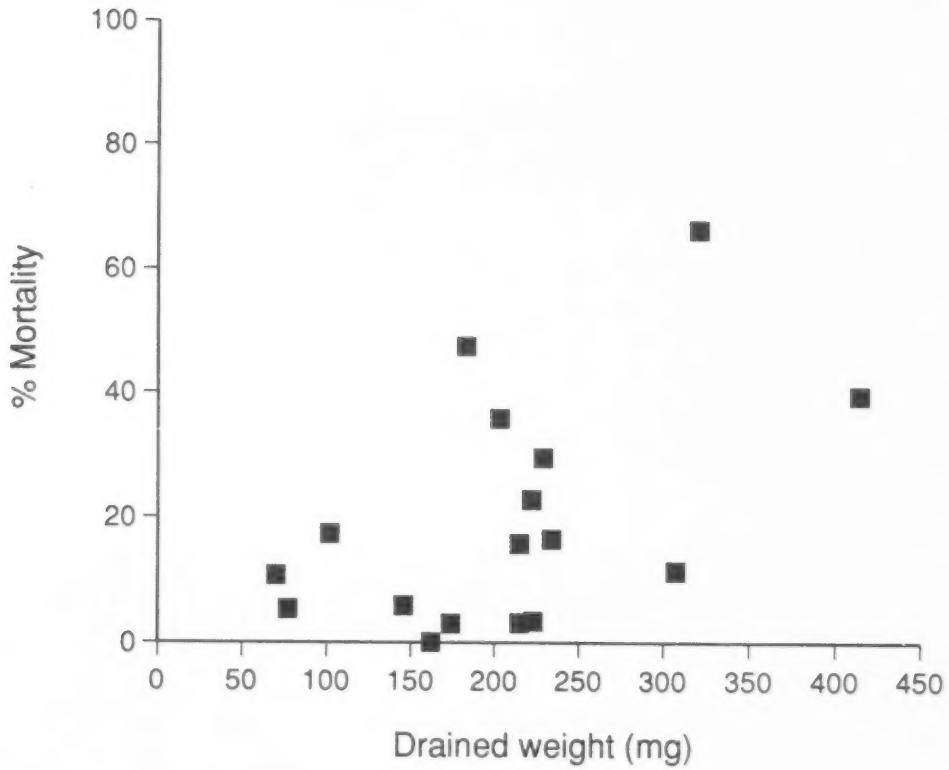
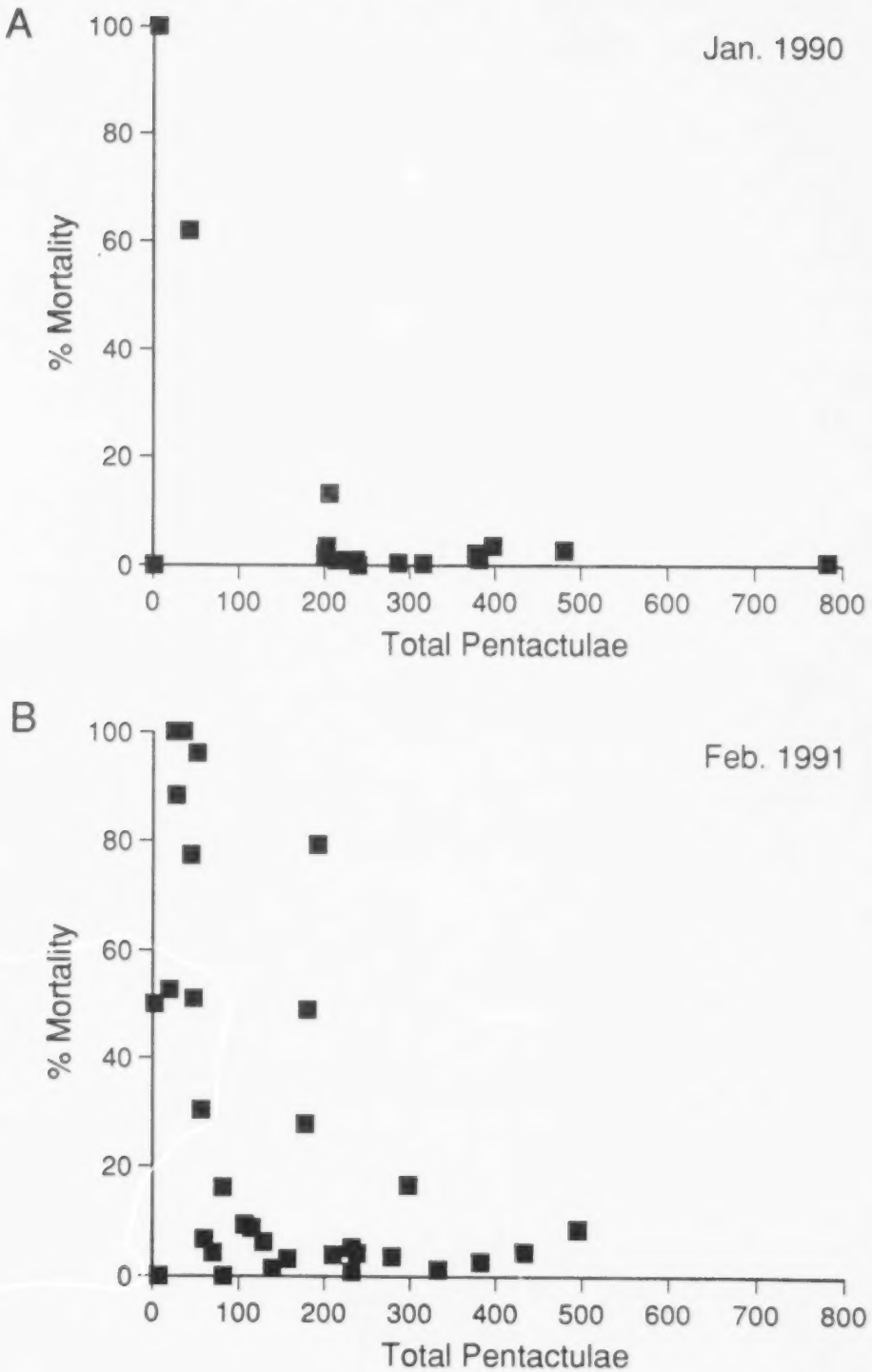

100
80
60
40
20
0
% Mortality
0
50
100
150
200
250
300
350
400
450
Drained weight (mg)

Figure 5-4. Relationship between total pentactulae number and percent mortality of pentactulae brooded in female Leptosynapta clarki in January 1990 and February 1991.


A
Jan. 1990
% Mortality
100
80
60
40
20
0
0
100
200
300
400
500
600
700
800
Total Pentactulae
B
Feb. 1991
% Mortality
100
80
60
40
20
0
0
100
200
300
400
500
600
700
800
Total Pentactulae

Two assumptions have, however, been made in the present study: a) that there is negligible mortality in the period prior to hatching of the pentactulae. In Leptosynapta clarki this is probably a valid assumption as the pentactulae hatch from the follicular membrane 1-2 weeks after fertilization (Chapter 2). Embryos, therefore, have a calcareous ring for ca. 93% of the 30 week brooding period (Chapter 2). b) This method assumes that there is limited resorption of calcareous rings within the ovarian tubule. There is currently no evidence to suggest that the calcareous rings are resorbed. In some dead pentactulae the connective tissue holding the pieces of the ring was resorbed but in all cases the calcareous pieces of the ring remained in the tubule.

By studying embryonic mortality in a brooding species we are able to examine mortality within a single female. While planktonic mortality rates by other authors (see reviews: Chaffee and Strathmann 1984, Strathmann 1985, Hines 1986, Rumrill 1990) are taken on a mixture of offspring from any number of females, in brooders the characteristics of the mother, such as size, may be directly related to the reproductive success of a specific female (number of embryos successfully brooded).

However, my data suggest no clear relationship between maternal features and reproductive success. Strathmann and Strathmann (1982) suggested that larger broods from larger adults might develop more slowly or suffer higher mortality during brooding. In Leptosynapta clarki while there is a trend for larger females to have a higher number of pentactulae, this relationship is weak and probably a consequence of problems with internal fertilization regardless of female size (Chapter 4). Although sample sizes are small, there is no consistent trend for a higher degree of mortality in large females or females with the largest number of pentactulae. In fact in the two samples with significant relationships, the highest percent mortality was observed in females brooding the fewest pentactulae.

While the data from Leptosynapta clarki suggest that mortality in a viviparous species can be extremely high, the reasons for death of pentactulae within the ovary are at present unknown. Pentactulae might die because of insufficient supplies of food or oxygen, genetic abnormalities, or the buildup of waste products within the ovary. Once dead, however, resorption of the dead pentactulae may be important in providing nutrition for the remaining pentactulae. Initially, nutrition for the pentactulae is provided from the breakdown of unfertilized eggs (Everingham 1961, McEuen 1986, Chapter 2). Later in the brooding period there are no discrete droplets visible in the tubules and the pentactulae feed on the fluid itself (Everingham 1961, Chapter 2). Resorption of the dead pentactulae may be important in providing additional organic material to the ovarian fluid.

While loss of the entire brood has been observed in other brooding echinoderms (Engstrom 1982, Strathmann et al. 1984), observations of non-brooding females might alternatively result from unsuccessful fertilization. In contrast, although eggs of some female Leptosynapta clarki are not fertilized during the spawning period (Chapter 2), the presence of calcareous rings in the tubules confirms that some female Leptosynapta clarki that were fertilized lose their entire brood.

Leptosynapta clarki shows values of instantaneous mortality of a similar order of magnitude to other marine invertebrates with benthic development (0.0046-0.0734 d^{-1} Rumrill 1990). However, as noted earlier, these comparative data need to be viewed with some caution because of assumptions about fertilization and the possibility of embryo release during sampling. Additionally, instantaneous mortality rates are probably not constant over the interval of observation in these studies (Chaffee and Strathmann 1984). Seasonal changes in mortality have been observed in Leptasterias tenera which broods slow-developing abortive embryos late in the season (Hendler and Franz 1982) and in the present study.

An additional source of mortality in brooding marine invertebrates not considered in a measure of instantaneous mortality results from mortality of the mother during the brooding period. For example, Asterina phylactica are consumed while brooding by the larger Asterina gibbosa (Emson and Crump 1976), presumably resulting in death of the brood. Female Leptasterias hexactis dislodged by water movement may also lose their entire clutch (Menge 1974), though this might not result in death of the female. In Leptosynapta clarki during periods of high rainfall and low intertidal salinity adult sea cucumbers were found dead on the sediment surface (unpub. obs.). Unless the pentactulae are released beforehand, death of the female will result in death of her brood as well.

The presence of an embryonic structure that is retained by the parent provides a method to study both fertilization and mortality in internal brooders. Preliminary data collected in Leptosynapta clarki suggests that mortality in a viviparous species can be extremely high, and questions the assumption that the lowest mortality will be found in species with the most advanced brood protection (Thorson 1950). The information available from this species is, however, insufficient to test Chia's (1974) hypothesis of decreasing mortality with increased brood protection.

Brooding sea cucumbers may provide better estimates of embryonic mortality than previous studies of brooding echinoderms (Menge 1974, Hendler and Franz 1982, Rumrill 1982, Strathmann et al. 1984) and allow a direct test of Chia's (1974) hypothesis. At least 41 species of holothurians brood their young, with 26 species brooding

externally or in pouches, 11 species brooding internally in the coelom and 4 species in the ovary or ovotestis (Smiley et al. 1991, Materia et al. 1991). Species chosen for such a comparative test should brood within a structure (pouch, ovary) or discrete area (e.g. under the female) that will retain the calcareous rings of any dead pentactulae. Comparing mortality in external brooders to coelomic and ovarian brooders such as Leptosynapta clarki could provide the basis for testing Chia's (1974) hypothesis in brooding sea cucumbers.

It is also possible that the present method may be applicable to measuring mortality in internal brooders such as gill-brooding bivalves and in the 13 other intraovarian brooding echinoderms (see Chapter 1). Messing (1984), for example, observed a sphere with the intraovarian embryos of the crinoid Comatilia iridometriformis that might represent an embryo undergoing resorption. Degenerating embryos observed fused to the internal surface of adult Ophionotus hexactis (Morison 1979) may have been too large to exit the bursae (Turner and Dearborn 1979), or alternatively may have died earlier in development. Careful dissections in such species to look for remains of embryonic structures might provide further estimates of mortality during brooding.

Because most species probably experience highest mortality during their early developmental period, Vance (1973a) concluded that "those forces affecting larval mortality probably play a very important role in the evolution of the general pattern of reproduction" (p. 339). Thus we should continue to collect information on larval mortality in species with a wide range of reproductive patterns. By focusing attention on encapsulating and brooding species we may be able to understand the importance of larval mortality in the evolution of life history strategy.

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CHAPTER 6

Birth, recruitment and juvenile growth in the intraovarian brooding sea cucumber Leptosynapta clarki.

A. Introduction.

The majority of sea cucumbers are broadcast spawners, with external fertilization and planktonic development. However, at least 41 species of holothurians brood their young under the sole, in the tentacles, in brood pouches or pockets, or internally in the coelom or ovary (Smiley et al. 1991). Although there is some information on life history characters and embryonic development in brooding species (Smiley et al. 1991), there is limited information on the events occurring immediately after juvenile release. For internal brooders, only one study has observed juveniles in the benthic habitat soon after release, and gives information on early growth (Engstrom 1980).

Leptosynapta clarki is an intraovarian brooding apodid holothurian found on intertidal mudflats on the west coast of Vancouver Island, Canada. During brooding, the fertilized eggs develop rapidly to the pentactula stage (1-2 weeks, Chapter 2) and remain in the ovary for 5-6 months (Everingham 1961, Chapter 2). Although the embryology of brooding in Leptosynapta clarki has been described (Everingham 1961) the subsequent fate of the juveniles after release has not been determined. In this study a recruitment pulse of newly released juveniles was observed on the mudflats in the late spring (April/May). Growth of the juveniles could be followed in the field until the end of the summer, when rapid growth made them indistinguishable from sea cucumbers of age > 1 year. Hereafter, laboratory growth experiments allowed growth to be measured until the sea cucumbers were 1 year old. This chapter provides the first detailed field study of growth of an apodid sea cucumber from the point of juvenile release, and represents a first step in our understanding of the early life history in Leptosynapta clarki after release of pentactulae from the ovary.

B. Materials and Methods.

1. Measurement of size.

Measurements of length (in mm) were made after relaxation of the sea cucumbers in 2.5% $MgCl_2$ in sea water (w/v) in a 90 mm petri dish. During relaxation the sea cucumbers would often retract or withdraw the tentacles. To standardise this

measurement, length was measured from the anterior end of the calcareous ring to the posterior while in the $MgCl_2$ relaxation solution. Animals less than 20 mm were measured on a dissecting microscope using an eyepiece graticule calibrated against a stage micrometer. Larger sea cucumbers were gently held straight with tweezers and length measured to the nearest millimetre with reference to a small plastic ruler under the petri dish.

After measurement of length, the sea cucumber was placed on a tissue to remove external water, and weighed on a fine scale balance to the nearest mg (total weight). There are some errors in this measure as the weight includes sediment and/or water in the alimentary canal. However, sea cucumbers left in sea water for removal of sediment tended to autotomize the posterior end, which led to greater inaccuracies in measurement of size.

2. Size of pentactulae prior to release.

Female Leptosynapta clarki were collected from Barge Bay, Grappler Inlet, Bamfield, British Columbia (48°49'57" N, 125°06'45" W) on 13 April 1990 (N=6) and during late March and early April 1991 (23 to 30-03-91 and 8-04-91, N=29). Females were relaxed in 2.5% $MgCl_2$ and the gonad dissected into a petri dish. Pentactulae were removed from the gonad tubules by gentle teasing with fine needles and size was measured as described above. In 1990 the pentactulae from four females were used to set up subcontainers 1-6 as a pilot study for the growth experiments (N= 38 pentactulae; see details below). Pentactulae from the remaining two females were used in pilot studies to test for recovery after relaxation in the $MgCl_2$ solution.

3. Recruitment and growth in the field.

Recruitment in Leptosynapta clarki was monitored in sediment cores from the mid-intertidal mudflat in Barge Bay (Site 1: 48°49'57" N, 125°06'45" W). Haphazard samples were taken using a perspex core (cross-sectional area= 78.54 cm^2) to a depth of ca. 10 cm (N=10 cores; Total area sampled = 785.4 cm^2). Core samples were combined in buckets and returned to the laboratory. Pilot studies found no sea cucumbers below 10 cm because of the shallow anoxic layer (unpub. data). Samples were taken every two months from May 1990 until August 1991.

In the laboratory, the sediment was sieved through a 2 mm or 0.85 mm sieve to remove adults, and the remaining fraction sieved through a 0.25 mm sieve to remove juveniles. Animals were kept in sea water for a maximum of 4 hours before measurement (length and weight). From May until August 1990, only juvenile sea

cucumbers were measured. Juveniles < 1 year old could be distinguished from older individuals as they were unpigmented. However, after August there was no clear distinction between age classes and measurements were made on all sea cucumbers in the sample. Weight of newly released juveniles in May 1991 was determined by regression (length vs log weight for animals 0-10 mm in length from the 15 July 1991 sample: log weight= 0.114(length) - 0.0197; r^2= 0.793, N= 33).

4. Laboratory growth of juveniles.

Growth experiments were conducted in two flat plastic trays (41.7 x 35.6 x 11.5 cm, L x W x D), subdivided into six approximately equal divisions by wooden partitions sealed at the edges with silicon sealant (i.e. each subcontainer ca. 12.5 x 15.5 cm). Each subcontainer was provided with air from an airstone glued to the base. Airstones were constructed from a 7.5 cm length of 1 mm borosilicate disposable pipette with three small holes on the upper surface. The pipettes were covered with 64 µm Nitex screening which provided slow diffuse aeration throughout each subcontainer.

Each container was filled to a depth of 7-10 cm with sediment collected from Barge Bay. This sediment was sieved through a 0.85 mm sieve to remove any sea cucumbers before placing in the experiment. However, when mud was added to the container to replace sediment lost as a result of aeration, additional juvenile sea cucumbers were sometimes introduced. These animals could be identified because of their small size and were ignored in the size-frequency analyses. The containers were immersed in a 1.7 x 0.72 x 0.22 m sea water tray with flow-through sea water. Water temperature throughout the experiment ranged from ca. 8-11^{o}C.

Juvenile sea cucumbers used in the growth experiment were obtained by dissection of the mother (subcontainers 1-6) as described above, or from field collections (subcontainers 7-12). Sediment was collected from Barge Bay on 14 May 1990, sieved through a 2 mm sieve to remove the adults, and then the remaining fraction spread in a shallow layer in trays and allowed to settle. Under bright light, reflection of the calcareous ring made juvenile Leptosynapta clarki visible on, or near the surface in shallow burrows. Juveniles were removed from the trays using a wide-bore pipette. Length was measured after relaxation in the $MgCl_2$ solution and the sea cucumbers placed in the sediment of the subcontainers.

Repeated measurements of size were made at 2-monthly intervals from May 1990 until May 1991. At such times sea cucumbers were removed from the sediment in each subcontainer using a 0.85 mm sieve, measured and returned to their respective subcontainers. The data presented are the pooled results of the two containers from 24

July 1990 until 12 May 1991. Although the experiment began in May 1990, the early results could not be used because of contamination of subcontainers 1-6 by additional juvenile sea cucumbers. In subsequent additions of sediment, special care was made to remove all sea cucumbers. The initial number of sea cucumbers in subcontainers 1-12 on 24 July 1990 was 245.

C. Results.

1. Field collections.

Pentactulae removed from the ovarian tubules in April 1990 ranged from 0.46 to 6.67 mm (mean ± SD= 2.00 ± 1.04 mm, N= 100). Pentactulae in most ovaries were less than 4 mm in length, with a modal size of 1-2 mm (Fig. 6-1). A similar size-frequency distribution was observed in late March 1991 (Fig. 6-2), although the sample was taken earlier and the mean length was lower at 1.24 mm (SD = 0.463 mm, N= 1735, range = 0.32-3.4 mm).

Birth of the pentactulae was never observed in nature, although in the laboratory normal handling of the female sea cucumbers during April and May would often result in the release of juveniles. Prior to release of the pentactulae from the coelom a break point occurs in the area between the brooding portion of the ovary and the basal region of renewed growth (Chapter 2). The posterior distal part of the ovarian tubule then becomes loose in the coelom (Chapter 2). During handling, the end of this tubule would often protrude out the body wall in the region of the anus. It is uncertain whether the tubule exited through the anus itself or through the body wall adjacent to the anus. Movement of the female resulted in the breaking of the thin ovarian wall and release of the juveniles. Female sea cucumbers were also very prone to autotomise during this period. Autotomy of the posterior end of the animal similarly exposed the tubules to the exterior and resulted in juvenile release. If sediment was provided, the juveniles rapidly formed shallow burrows.

After release from the mother at a length of 1-2 mm (Fig. 6-1, 6-2), rapid growth was observed in the field to a maximum of 5.00 mm in mid-May 1990 (Fig. 6-1) and 9.13 mm in late May 1991 (Fig. 6-2). By July, the modal length was 5-6 mm, with a maximum of 13.78 mm in 1990 (Fig. 6-1), and 14.77 mm in 1991 (Fig. 6-2). There is considerable variability in growth during the late summer, so that by August the juveniles are generally 6-16 mm in length (Fig. 6-1, 6-2).

Release of juveniles into the sediment could be observed as a recruitment pulse into the adult population in Barge Bay (Fig. 6-3). The juveniles released in April/May

Figure 6-1. Size-frequency distributions of Leptosynapta clarki pentactulae prior to release (grey bars) and during growth in sediment (black bars) in 1990.

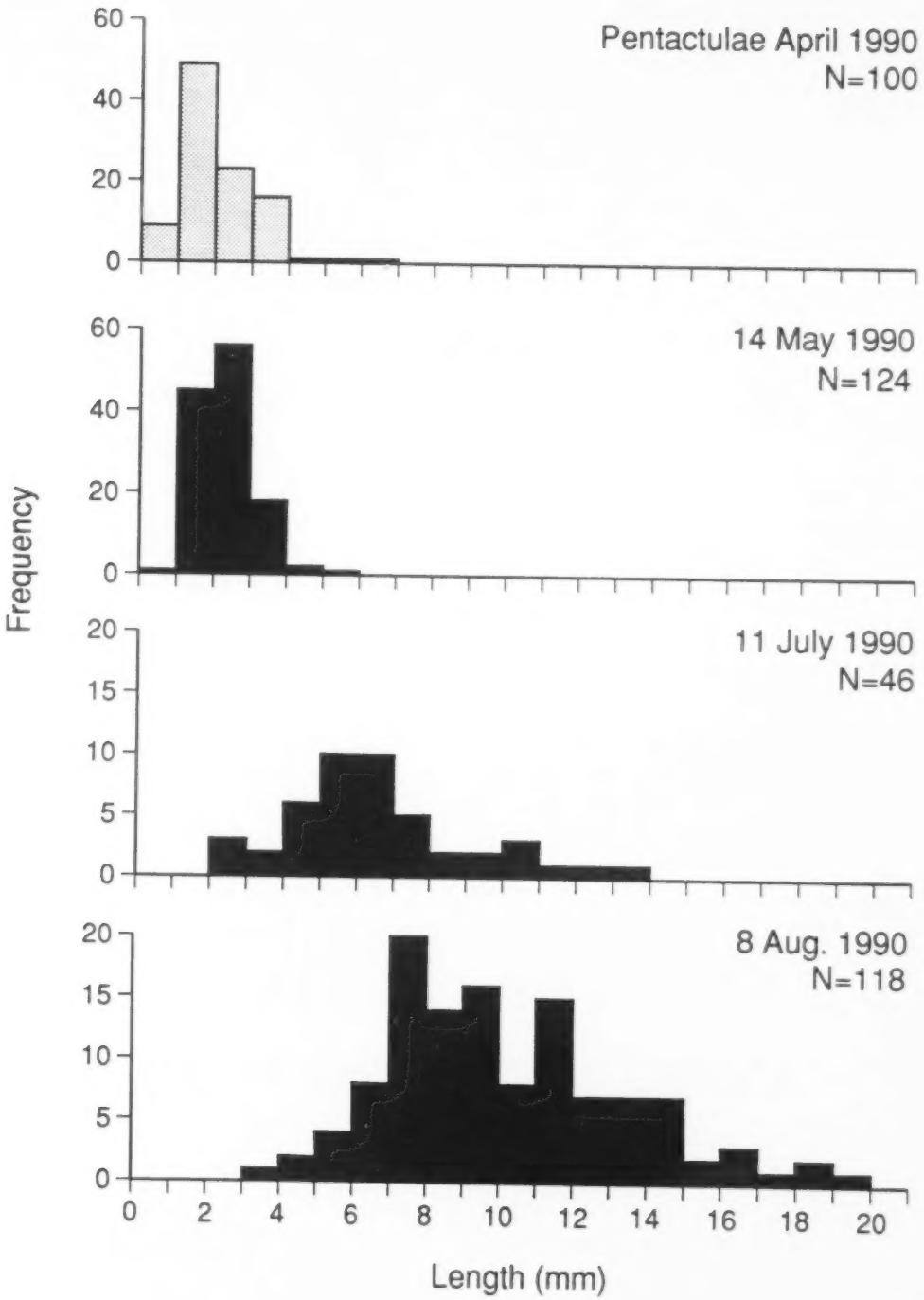

Pentactulae April 1990
N=100
14 May 1990
N=124
11 July 1990
N=46
8 Aug. 1990
N=118
Frequency
Length (mm)
0
20
40
60
5
10
15
2
4
6
8
12
14
16
18

Figure 6-2. Size-frequency distributions of Leptosynapta clarki pentactulae prior to release (grey bars) and during growth in sediment (black bars) in 1991.

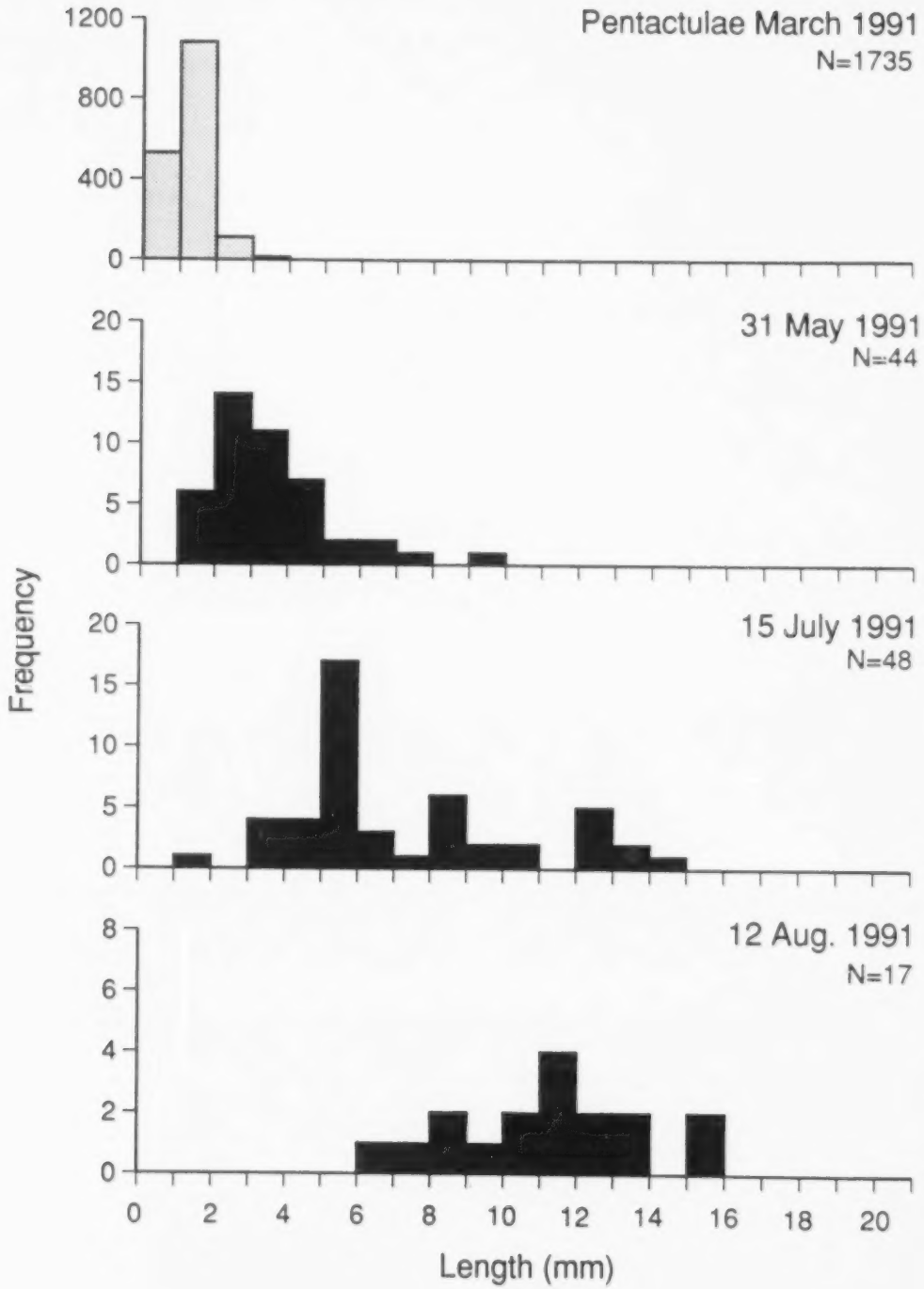
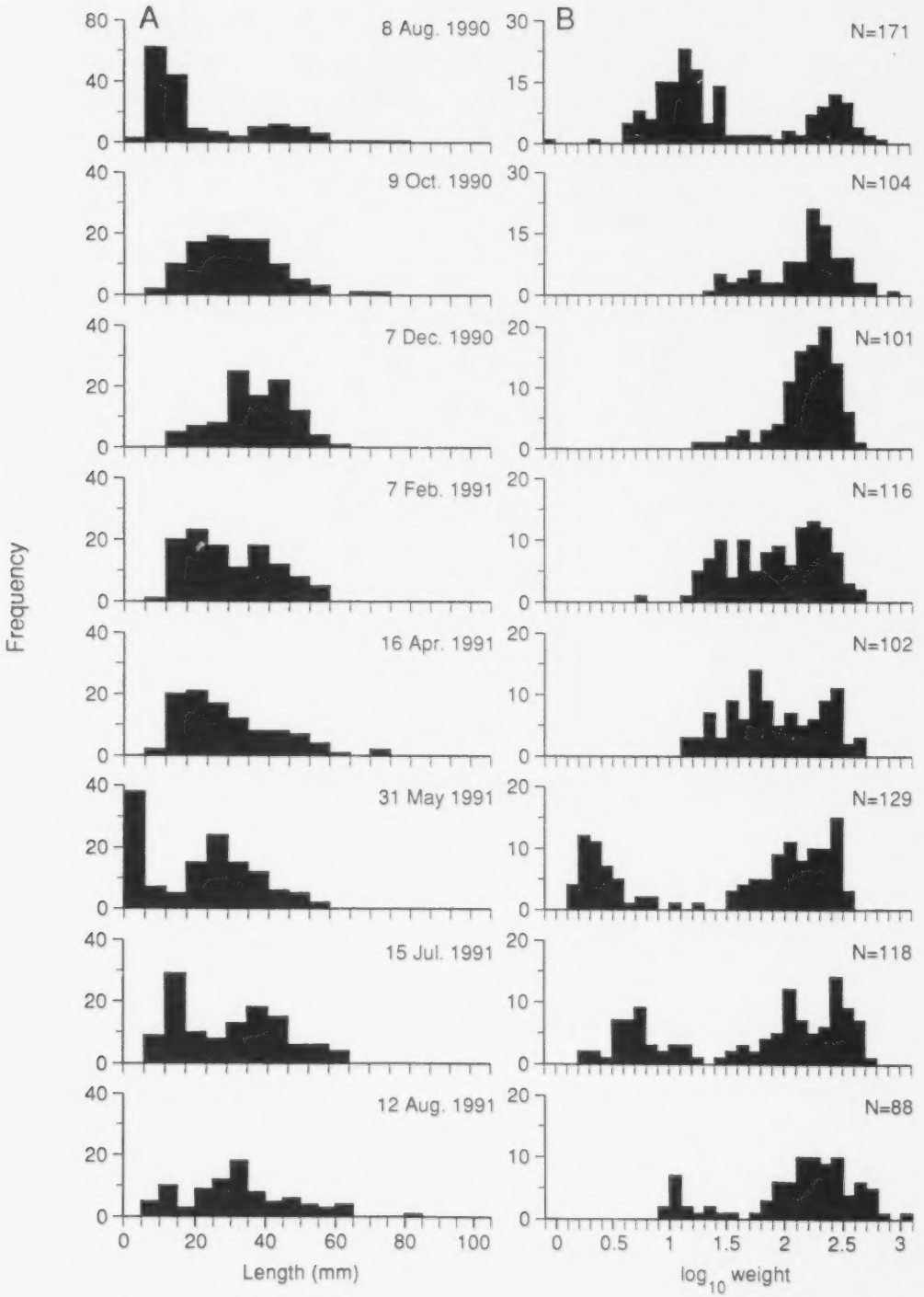

Pentactulae March 1991
N=1735
31 May 1991
N=44
15 July 1991
N=48
12 Aug. 1991
N=17
Frequency
Length (mm)
1200
800
400
0
20
15
10
5
0
20
15
10
5
0
8
6
4
2
0
0 2 4 6 8 10 12 14 16 18 20

Figure 6-3. Size-frequency distributions of Leptosynapta clarki in Barge Bay from August 1990 to August 1991. A. Size measured as length (mm). B. Size measured as $\log_{10}$-weight (original data in mg). Dates of collection are shown in A; number of sea cucumbers in sample in B.


A
B
8 Aug. 1990
N=171
9 Oct. 1990
N=104
7 Dec. 1990
N=101
7 Feb. 1991
N=116
16 Apr. 1991
N=102
31 May 1991
N=129
15 Jul. 1991
N=118
12 Aug. 1991
N=88
Frequency
Length (mm)
log10 weight

1990, were still visible as a distinct cohort in both length and weight in August (Fig. 6-3). By October, the juvenile cohort was indistinguishable from the adult population in length (Fig. 6-3A), and remained so throughout the autumn and winter. There were, however, a number of indistinct modes in weight (Fig. 6-3B). Newly released juveniles are clearly visible as a recruitment pulse in May 1991 (Fig. 6-3). This new cohort showed rapid growth through the summer (Fig. 6-2), and was still visible as a distinct cohort in both length and weight in August 1991 (Fig. 6-3).

The density of Leptosynapta clarki in the Barge Bay population was fairly constant throughout the period of sampling (mean ± SD: 168.8 ± 34.6 m^{-2}, N= 8). No consistent changes in density were noted throughout the year as both the lowest density (124.8 m^{-2}) and the highest density were found in August (244.5 m^{-2}). Density estimates of Leptosynapta clarki in Barge Bay may, however, be affected by the patchy distribution of juveniles (unpub. obs.).

The largest individual observed at this site was 82 mm in length and 1060 mg in weight. Specimens of Leptosynapta clarki do, however, attain larger sizes at other sites in Grappler Inlet. The largest specimen found at Bamfield had a length of 113 mm and weighed 1291 mg (Chapter 4).

2. Laboratory growth of juveniles.

Growth of Leptosynapta clarki in the laboratory was similar to that observed in the field collections (Fig. 6-4, Table 6-1), but growth could be monitored after August when the Year 1 juveniles were indistinguishable from adults in field samples. In late July 1990, juveniles in the laboratory showed a mode at 6-7 mm (Fig. 6-4), which was similar to that in the field (Fig. 6-1), although with a larger variability. Juveniles continued to grow rapidly until they reached a maximum of 30 mm by September (Fig. 6-4), and a mean weight of 23 milligrams (Table 6-1). No laboratory measurements were made in August to compare with the field collections, but the mean for the August field sample (mean ± SD: 10.22 ± 3.20 mm, N= 118) is intermediate between the laboratory means for July and September (Table 6-1). Based on the 1990 field collections, it appears that juvenile growth estimates from the laboratory are in the same range as those found in natural populations.

At the time of spawning of Leptosynapta clarki in November/December (Chapter 2), the maximum length of young of the year was 35.5 mm (Fig. 6-4, Sewell 1991). This particular individual was 200 mg total weight. In many of these Year 1 sea cucumbers white testes were observed through the body wall (10 Dec 1990 sample, 65 of 120 were identified as males). Dissections of sea cucumbers in this size range confirmed that

Figure 6-4. Laboratory growth in Leptosynapta clarki until an age of approximately 1 year. Size-frequency distributions in length (mm) are pooled results from subcontainers 1-12.

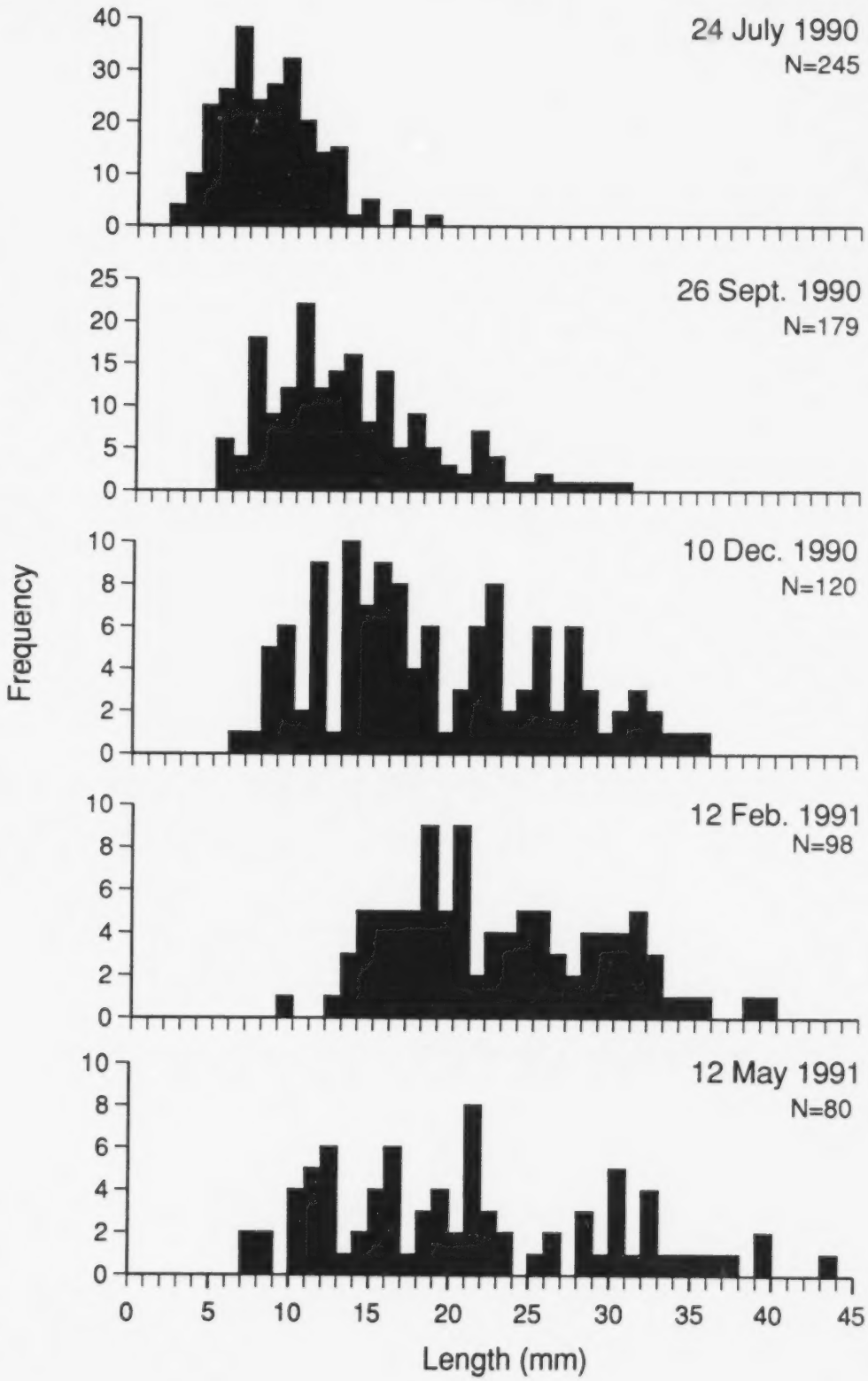

24 July 1990
N=245
26 Sept. 1990
N=179
10 Dec. 1990
N=120
12 Feb. 1991
N=98
12 May 1991
N=80
Frequency
Length (mm)

Table 6-1. Laboratory growth in Leptosynapta clarki. No weight measurements were made at the start of the experiment due to small size. Size-frequency data for this experiment are shown in Fig. 6-4.

	Length (mm)		Weight (mg)		
DATE	Mean	S.D.	Mean	S.D.	N
24 July 1990	8.19	3.04	-	-	245
26 Sept. 1990	13.37	5.13	23.0	18.6	179
10 Dec. 1990	18.73	6.95	53.4	46.3	120
12 Feb. 1991	19.61	6.27	50.2	31.1	98
12 May 1991	21.08	8.72	74.7	63.1	80

Leptosynapta clarki have active sperm and are reproductively active as males for their first reproductive season.

By the end of the first year, the mean size of juveniles was 21.1 mm length and 74.7 mg weight (Table 6-1). The largest juvenile was 44 mm in length (Fig. 6-2), and 261 mg in weight.

There is considerable variability in growth rate which is partly due to food availability, but is also related to unintentional damage during measurement. The small size and delicate nature of the body wall made it difficult to remove the animals after sieving. Juveniles also autotomize the posterior end which decreases the length and weight. Damage, poor conditions, and loss of some animals in measurement lead to a gradual decline in the number of sea cucumbers. Numbers were reduced from 245 to 80 (Table 6-1); an overall recovery of 33%.

D. Discussion.

Two principal methods have been described for the release of embryos from the coelom of internally brooding holothurians: (a) By rupture of the body wall (Leptosynapta clarki, Everingham 1961, present study; Staurothyone inconspicua, Materia et al. 1991; unidentified cucumariid, O'Loughlin 1991) or (b) by escaping through the anus, either by rupturing the rectal wall (Synapta vivipara, Clark 1898; Pachythyone rubra, Chaffee 1982; Lissothuria antillensis, Miller 1985) or via anal pores (Leptosynapta clarki, Brooks 1973). Embryos are also suggested to escape through rectal or abdominal pores in Leptosynapta minuta (Becher 1912, in Ohshima 1916) and Synaptula hydriformis (Turner 1973), and may escape through the anus in Neoamphicyclus lividus (Materia et al. 1991). Opportunistic release of embryos can also occur during autotomy (Brooks 1973, present study), evisceration (Chaffee 1982) or handling (Materia et al. 1991).

Once juvenile Leptosynapta clarki are released from the mother they burrow into the sediment immediately and are visible as a distinct recruitment pulse in the adult habitat. However, while not directly examined in this study there is anecdotal evidence for subsequent dispersal of juveniles from the site of release. This dispersal may occur by rafting, floating, swimming, or incidental dispersal as a result of currents or wave action.

Rafting has been observed in a single Leptosynapta clarki in Mitchell Bay, San Juan Island, Washington on an Ulva-Monostroma algal mat (Highsmith 1985). During the spring and summer Leptosynapta clarki at Barge Bay are also found within

filamentous Enteromorpha spp. mats on the surface of the mudflat. Outgoing tides from Barge Bay include clumps of this alga (pers. obs.) and therefore algal rafting might provide a method for dispersal in both juvenile and adult L. clarki (Highsmith 1985).

More passive dispersal might occur if the juveniles can float suspended on the water surface (sensu Highsmith 1985) as noted in juvenile synaptids (Clark 1907). Juveniles of Leptosynapta clarki were often caught in the surface tension in the laboratory, and if present on the mud surface during outgoing tides could float away from the natal site. The finding of a juvenile Leptosynapta clarki in a sediment collector in Barge Bay 10 cm above the surface and another in an oblique plankton tow in the mouth of Grappler Inlet (unpub. obs.) suggests that there is some potential for dispersal. However, the frequency and importance of floating for dispersal in this species is as yet undetermined.

Additionally, many apodids are observed to be nocturnal swimmers at restricted times of the year (Leptosynapta inhaerens, Costello 1946; Labidoplax dubia, Hoshiai 1963; Leptosynapta albicans, Glynn 1965). In all cases the sea cucumbers had empty or reduced amounts of sediment in the gut during swimming (Costello 1946, Hoshiai 1963, Glynn 1965). While the velocity of swimming is slow (5-6 cm/min in juveniles, Costello 1946; ca 1 m/min in adults, Glynn 1965) if aided by water currents the sea cucumbers might be able to disperse over a reasonable distance.

Finally, incidental dispersal of juveniles and adults may occur due to currents or wave action. Engstrom (1980) noted that Chirodota rotifera if starved or in foul water would empty the gut of sediment and move actively on the substrate surface. This behaviour made the animals nearly neutrally buoyant and Engstrom (1980) suggested they could be easily transported by tidal currents or mild wave action. Wave action may also sweep juveniles of brooding intertidal species into deeper water (Pseudocucumis africanus, Ohshima 1916; Cucumaria pseudocurata, Rutherford 1973, Martel 1990) or lift burrowing species to the surface during severe storms (e.g. adult Leptosynapta inhaerens, Fish 1925).

Dispersal of either juvenile or adult Leptosynapta clarki may be important in assuring random mating within and between populations. Electrophoretic studies of Leptosynapta clarki on San Juan Island, Washington, by Hess et al. (1988) found there was enough movement of either individuals or gametes within False Bay to allow random mating and maintain heterozygosity within populations. However, differences in the genetic composition between geographic locations suggests that although gene flow between L. clarki populations is probably rare, it does occasionally occur (Hess et al. 1988). Gene flow could result from the dispersal of juveniles which introduces new

males into a population, or as adults. In the latter case, dispersal of brooding female L. clarki might introduce large numbers of individuals into a population allowing genetic mixing or colonization of suitable habitat.

While the potential for dispersal in brooding holothurians deserves more attention the fate of the juveniles once they reach the benthic habitat is virtually unknown. Although recruitment has been studied in holothurians with planktonic larvae (see Ebert 1983, Muscat 1983, Cameron and Fankboner 1989), there are only a few studies that report finding juvenile apodids (Menker 1970, Rao 1973, Engstrom 1980) and only one study of recruitment in a brooding sea cucumber (Cucumaria pseudocurata, Rutherford 1973). The latter study, although only one month in duration, provides the only directly comparable study of recruitment in a brooding sea cucumber.

In brooding species because the juveniles are usually released over a short period of time and the numbers of recruits are not subject to the vagaries of planktonic transport we might expect a pronounced increase in the number of juveniles during the period of release. This pattern has been observed in Cucumaria pseudocurata after juveniles crawl from under the mother (Rutherford 1973) and in Leptosynapta clarki after pentactulae release. In the present study there was, however, no dramatic decrease in numbers of juvenile L. clarki in the first month as observed in Cucumaria pseudocurata (Rutherford 1973). While there must be some juvenile mortality in L. clarki, in the present study the juvenile cohort could be followed for approximately 6 months until it was incorporated into the adult population. This, therefore, provides the first long-term study of recruitment in a brooding holothurian and in any apodid sea cucumber.

Comparative information on growth rates in brooding sea cucumbers is similarly limited. Growth estimates are available for only two species: the external brooder Cucumaria pseudocurata (Rutherford 1973) and the coelomic brooder Chirodota rotifera (Engstrom 1980). There is, however, some qualitative evidence for rapid growth after release in three other apodids: Synapta vivipara Clark (1898), Labidoplax buskii (Nyholm 1951), and Rhabdomolgus ruber Menker (1970).

Once in the sediment juvenile Leptosynapta clarki grow very rapidly from a length of 1-2 mm to a mean length of 21 mm in their first year. In L. clarki, as in juvenile Cucumaria pseudocurata (Rutherford 1973), this rapid growth occurs during the summer months. The rate of growth observed in L. clarki is comparable to that seen over a shorter period of time in the apodid Chirodota rotifera by Engstrom (1980). Four C. rotifera juveniles collected in sand from Key Biscayne, Florida, and maintained in laboratory culture for 3 months grew from 1.5 mm to 10 mm in length before death (Engstrom 1980). As the largest pentactula found in C. rotifera from Jamaica was 3 mm

(Clark 1910), this suggests that Engstrom (1980) cultured recently released juveniles. If this rate of growth had continued these C. rotifera would probably have attained a similar size to Leptosynapta clarki in their first year.

By the age of one year, Leptosynapta clarki have already reproduced as males in the early winter (Nov./Dec., Chapter 2). Two other apodids show rapid growth and early sexual maturity. The burrowing Labidoplax buskii is sexually mature after one year (Nyholm 1951) and the interstitial Rhabdomolgus ruber has gonads within 4-5 months of settlement (Menker 1970). In the latter species it is, however, unclear whether these individuals reproduce (Menker 1970).

By regular field sampling the population dynamics of Leptosynapta clarki could be followed in Barge Bay from the point of juvenile release. The use of two measures of size (length and $\log_{10}$weight) has allowed the juvenile cohort to be distinguished from adult sea cucumbers in field collections for appoximately 6 months after release. However, in the absence of distinct year classes in adults and the inability to determine the age of individuals, the rate at which L. clarki individuals move through the population from recruitment to death is unknown.

The method developed herein where sea cucumbers could be maintained in the laboratory, with growth rates approximating those in natural conditions, will be a useful tool for future population studies in small burrowing sea cucumbers. For those species in which tagging or marking is inappropriate because of size, a combination of laboratory experiments and field recruitment data may give us a greater insight into the early life history of many smaller sea cucumber species.

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CHAPTER 7

General Discussion.

The reproductive biology of the viviparous sea cucumber Leptosynapta clarki has been examined in Bamfield, British Columbia. While in general terms reproduction is similar to other annually reproducing holothurians, the reproductive cycle in Leptosynapta clarki is complicated by two features that may be related to its small adult size: brooding and protandry.

The most profound difference in reproduction results from the retention of the gonad tubules after spawning. This results in part of the previously fecund tubule remaining for the following reproductive season. In fertilized female Leptosynapta clarki the ovarian tubules are filled with pentactulae and remain at a large size until the breaking of the distal portion of the ovary in pentactulae release. The basal end regrows oocytes for the coming reproductive season. Similarly, in male L. clarki the previously fecund tubule is rarely completely resorbed after spawning and the regressed bud regrows new spermatogenic tissue. Most dramatically, however, is the retention of the post-spawned transitional testis where active oogenesis gradually fills the testis lumen with oocytes and the sea cucumber reproduces in the following season as a female.

The original questions posed in the introduction (Chapter 1) have been addressed in the various chapters of the thesis resulting in a better understanding of the reproductive biology of Leptosynapta clarki. In terms of reproduction, the only unanswered question in this study and the most important area for future research is the biology of spawning and fertilization.

The spawning behaviour of Leptosynapta clarki is still unclear. Aggregations of sea cucumbers, such as those observed at low tide during the spawning period of L. clarki at False Bay (Everingham 1961, McEuen 1988) were not observed at Bamfield. Furthermore, these authors were unable to provide evidence that these groups are in fact formed by spawning individuals. As natural spawning in two other apodid sea cucumbers occurs when water covers the burrows it is possible that intertidal L. clarki will spawn at periods other than low tide. By keeping L. clarki in the laboratory in approximately natural conditions observations on spawning might be made.

Everingham (1961) suggested that self-fertilization might occur in Leptosynapta clarki. The present study has clearly determined that the timing of fertilization (Nov.-Dec.) and the period of protandric sex change (Jan.-May) do not overlap. This means

that mature eggs and sperm are not present in the same gonad at one point in time, and self-fertilization is unlikely. Genetic evidence from Leptosynapta clarki on San Juan Island also found no evidence for self fertilization or inbreeding within the studied populations (Hess et al. 1988).

Leptosynapta clarki might be suitable for studying fertilization success in a brooding species . Preliminary results suggest that fertilization is variable with some females having a poor percent fertilization, while in others almost all the eggs are fertilized. Little is known of natural rates of fertilization in marine invertebrates (see Sewell and Levitan 1992), but especially in brooding species. Quantification of fertilization success in such brooding species would allow testing of assumptions such as those of Chia (1974) that problems with fertilization are reduced in brooding and viviparous species. Leptosynapta clarki is ideal for such a study as fertilized and unfertilized eggs are retained in the ovary, and the percent fertilization can be readily determined. These values of percent fertilization could then be compared to results from other brooding species.

Once the mechanism of fertilization in Leptosynapta clarki is understood we may be better able to determine the role size plays in reproduction in this species. The size-advantage hypothesis predicts that sequential hermaphroditism will occur where an individual reproduces most efficiently as a member of one sex when small or young, but as a member of the other sex when it gets older and larger (Ghiselin 1969, Charnov 1982). The 1:1 sex ratio seen above a weight of 500 mg in L. clarki is contrary to sex allocation theory but has been seen in three limpets (Branch 1981, Wright 1989, Creese et al. 1990). This pattern might be more explicable if the reproductive success of Leptosynapta clarki of a range of sizes could be determined. Preliminary evidence in Chapter 4 suggests that there is no relationship between female size and fertilization success but there is presently no method whereby the fertilization success of male Leptosynapta clarki can be determined. If spawning could be induced in the laboratory this would provide an interesting area for further experimentation.

Leptosynapta clarki shows the association of brooding and hermaphroditism as seen in the reproductive patterns of many small marine invertebrates. The allometry hypothesis for the association of brooding and small adult size predicts that larger females can successfully brood a smaller number of embryos because of space limitations (Strathmann and Strathmann 1982). A test of this hypothesis in female Leptosynapta clarki did not, however, indicate either spatial constraints on brood size or differential embryonic mortality in larger females. This might result from problems in fertilization

regardless of size, or because brooding occurs in a distensible structure that can expand to accommodate large numbers of pentactulae.

A second important area for further research is the mechanism of sex determination in Leptosynapta clarki. Growth studies in the laboratory and field recruitment studies have shown that all Year 1 individuals reproduce as males approximately 6 months after their birth. The sex of that individual in subsequent years is not simply a function of the size or age of the individual but seems to depend on the interaction of additional factors. If there are two sexual races within a population the genetic predetermination for one sex or the other may be also influenced by environmental factors as yet unmeasured.

With respect to protandry, two questions remain: 1) do some individuals remain as males for their reproductive life?, i.e. never change sex and 2) is it possible for females to reverse the direction of sex change and return to reproduction as males? Answering these questions will probably be difficult. Firstly, while sex can be determined in the autumn without dissection, by visualising the gonad through the semi-transparent body wall, a method would need to be developed to individually mark sea cucumbers over 2 to 3 years to determine the sequence of sex in each year's reproduction. Tagging of large sea cucumbers has been successful in two temperate species (Muscat 1983, Da Silva et al. 1986) but not in seven tropical species (Conand 1991) and may be impossible in smaller sea cucumbers. Alternatively, adult sea cucumbers could be maintained over their reproductive life in individual containers or cages in the laboratory or field. However, there are two potential problems with this approach. Firstly, adult Leptosynapta clarki maintained in the laboratory shrink in size (unpub. data) which would confound the test as size is important to reproducing as a female. Secondly, the ability of adult L. clarki to escape through Nitex screen as small as 560 µm would make construction of cages that do not affect environmental conditions extremely difficult.

The general structure of the Leptosynapta clarki ovary is similar to that descibed in other holothurians. The structural changes in the ovarian wall that occur prior to fertilization are, to my knowledge, undescribed in echinoderms. Electron microscopy has shown that these changes in texture and wall thickness are not the result of any additional cell types but result from changes in the size and shape of existing cells of the ovarian wall. The functional significance of this structural change is as yet undetermined. Until comparative studies are conducted on broadcast spawning and viviparous sea cucumbers we will not be able to determine if these changes are related to the additional function of the ovary as a brood structure.

A significant finding in this study was that there may be extensive mortality of pentactulae during the brooding period. The retention of the calcareous ring after pentactulae death allowed accurate counts to be made of dead pentactulae and provided the first estimate of embryonic mortality in a viviparous marine invertebrate. The finding of high embryonic mortality in some female Leptosynapta clarki questions the assumption that there is low mortality in viviparous species. The generality of this finding, however, awaits further research on other brooding species.

For the first time in an apodid sea cucumber we have been able to measure growth after release of pentactulae from the mother and recruitment to the adult habitat. In combination with laboratory experiments this has allowed a detailed study of the age and size of sexual maturity and some information about the timing of sex change. The absence of distinct modes in the size-frequency distributions, except immediately after juvenile release, has however prevented estimates of growth in older animals.

Based on the information presented throughout the thesis we are able to estimate longevity of individual Leptosynapta clarki. As all Year 1 individuals are male, some males undergo protandric sex change after their first year, and there is evidence that females reproduce again as females, then individual Leptosynapta clarki probably live at least 3 years. However, as most juveniles in the laboratory were male for a second reproductive period, individual longevity may be 4 or 5 years. Leptosynapta clarki therefore confirms to the suite of life history traits associated with small adult size (Strathmann 1985), i.e., lower fecundity (max. 4739 eggs), early sexual maturity (6 months), lower adult longevity (ca. 5 years) and shorter generation time.

The present study has addressed questions on viviparity in Leptosynapta clarki in terms of life history theory. Information about brooding, protandry and embryonic mortality have been considered in light of appropriate theory. By taking this approach the information presented has applicability to other viviparous species rather than just to Leptosynapta clarki itself. As little information is available on viviparous species it is hoped that the present study acts as a catalyst to future research.

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